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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) This project was proposed to study the correlation between caspase deficiency and breast cancer carcinogenesis and therapeutic resistance. In the second year of our project, we continued to detect caspase expression in breast cancer derived cell lines. We have examined the expression of 9 caspases in about 20 breast cancer cell lines. Lower expression of certain caspases, especially for caspase 6 and 8, have been detected in certain cell lines. We also made total RNA from 19 cell lines for caspase expression analysis. We have performed apoptotic analysis of 11 breast cancer cell lines treated TNF- α using DAPI staining and 19 cell line treated with doxorubicin using MTT assay. More functional screening is underway. Using our caspase 3 deficient/ reconstituted MCF-7 cell line model, we characterized apoptosis induced by etoposide. The data has been incorporated in our previous manuscript reporting doxorubicin induced apoptosis. Studies on the role of caspase 3 in radiation induced apoptosis suggested that caspase 3 had a strong feedback action on mitochondria in radiation induced apoptosis, and that cleavage of lamin B and actin was more susceptible to radiation induced apoptosis. In addition, we also studied the interaction between caspase 3 and p53. One reprint, on manuscript and one thesis have been generated.				
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Introduction

Apoptosis (programmed cell death) is a fundamental process involved in homeostasis and the biological response to anti-tumor therapy. Aberrant expression of apoptotic regulators, such as Bax-a, Bcl-2 and p53 has been associated with breast cancer carcinogenesis and therapeutic resistance. Apoptosis is mediated by caspases, groups of proteases. We and others have shown that caspase 3, an effector caspase, is deficient in MCF-7 breast cancer cell line. This project was designed to study the incidence and significance of caspase deficiency in breast cancers. The three specific aims were:

1. To determine the incidence and pattern of caspase deficiencies in breast cancer via screening for specific caspase expression in breast cancer derived cell lines, explant cultures and snap frozen human breast cancer tissue.
2. To correlate caspase data (obtained in aim 1) with apoptosis induction using immune modulators (TNF-a, anti-Fas, GrB/Ad), chemotherapeutic agents and radiation on breast cancer derived cell lines and explant cultures.
3. To define the biologic role of specific role of caspase deficiencies via reconstitution of deficient caspases and comparative studies to define apoptosis induction in response to immune modulators chemo- and radiation therapy.

In the second year of this project, we continued to detect the expression of caspases in breast cancer cells and performed functional screening of these cells in response to TNF-a and doxorubicin. Meanwhile, we pursued further on the investigation of the role of caspase 3 in chemotherapy and radiotherapy mediated apoptosis and the results have been published in Cancer Research, which is followed by another manuscript and a student thesis.

Body

The research in the second year covered all 4 tasks proposed in the original proposal. The detailed progress is described as the following.

A. Studies on the role of caspase 3 deficiency in chemotherapy and radiotherapy mediated apoptosis

Studies in this part generated high productivity in our research. Although this belongs to task 4 in the original Statement of work, it could not be delayed because of the significance of our finding and the rapid development in the research of this area.

Last year, we submitted our paper “Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin induced apoptosis” to Cancer Research. While underscoring the significance of our research, the reviewer asked us to expand our study by including other drug treatment. As detailed in Appendix D, we performed detailed study on etoposide mediated sensitization in caspase 3 reconstituted MCF-7 cells and characterized nuclear morphologic changes of drug treated MCF-7 cells. This study has been published in Cancer research (Appendix D).

We also had some preliminary data on radiotherapy induced apoptosis in caspase 3 reconstituted MCF-7 cells from previous work, which was presented in 91st AACR meeting. To facilitate the publication of our results, we continued this study. Results from the second year indicated that cytoskeletal proteins, such as lamin B and actin, were more susceptible to radiation induced apoptosis and caspase 3 activity enhanced this process significantly. In addition, we also provided direct evidence that caspase 3 had strong feedback activity on mitochondria, which could be part of the sensitization mechanism (Detailed in Appendix B).

In the past year, Ms. Alison Johnson from the Master Program of Biotechnology at Northwestern University did her academic research in our lab. The research was on the role of p53 in caspase 3 activation during chemotherapy induced apoptosis in MCF-7 cells. As detailed in Appendix C, we studied the correlation between p53 function and caspase 3 activation by examination of endogenous p53 induction in drug treated MCF-7/pv and MCF-7/c3 cells, and by transfecting of dominant negative mutant (DNM) p53 in

both cells lines. We have established double transfectant MCF-7 cells expressing caspase 3 and dominant negative p53. Preliminary data suggest that introducing DNM p53 affected the activity of p21/waf-1 and caspase 3 level/activity in MCF-7/c3 cells. Further studies will be followed.

B. Continuation of Task 1 to detect caspase expression in commonly used breast cancer cell lines and breast cancer derived explant cell.

In the first year of the project, we had established detection conditions for different caspases and detected the expression of caspases 3, 6, 7, 8, 9, and 10 in MDA-MB-157, BT-20, HS 578T, MDA-MB-436, MDA-MB-435s, T47D, MDA-MB-453, MDA-MB-2312 and MDA-MB-468 breast cancer cell lines. This year, we continued to detect the expression of caspases 1, 2 and 4 in above 9 cell lines (Fig. 1, appendix A). We also detected the protein levels of caspases 1, 2, 3, 4, 6, 7, 8, 9 and 10 in BT-474, BT-483, MDA-MB-330, MDA-MB-415, MDA-MB-134VI, MDA-MB-361, ZR-75-1, ZR-75-30 and SK-BR-3 breast cancer cells (Fig. 2, Appendix A). Caspase 5 was not detected was due to the unavailability of appropriate antibody.

Analysis of the expression patterns of 9 caspases in 18 breast cancer cell lines revealed that expression of each caspase indeed varied from one cell line to another. The fluctuation of caspase levels among these cell lines was cell line and caspase dependent. For example, caspase 8 level was low in MDA-MB-415, MDA-MB-134 and ZR-75-30 cells, but it was high in BT-474 and MDA-MB-361 cells. MDA-MB-134Vi cells had relatively lower level of caspase 8 but had relatively high levels of caspase 10 (Fig. 2, Appendix A). Horizontal comparison suggested that variation of caspases 7 and 9 levels among different cell lines was not as obvious as the variation of caspases 6 and 8. Vertically comparison suggested that MDA-MB-415 (Fig.2 Appendix A) and HS-578T (Reported the last year) had relatively lower levels of most caspases.

Since the affinity of our antibodies against caspases 1 and 4 was so low that the real expression levels of these two caspases need to be verified when good antibody available.

For the rest of caspases, it is interesting that, although lower caspase levels were detected in different cell lines, we have not detected any cell line has completely deficiency of certain caspase as caspase 3 in MCF-7 cells. However, caspase 3 deficiency was detected in a Hodgkin's disease cell line KMH2 (1). Within a smaller sample pool, it appears that looking only at caspase expression levels by itself yield limited information. Correlation of caspase levels with functional assay (in task two) would be more meaningful.

We also extracted total RNA from 20 breast cancer cell lines (Fig.3 in Appendix A showing RNA gel of 17 samples). Growing 20 cell lines to make enough RNA took a great effort. The purpose of RNA extraction was not only for detecting caspase expression at RNA level. Given the complicated interactions among apoptotic regulators in different pathways, we hope we could also detect other apoptotic regulators by RT-PCR, which may help us to explain the association between caspase expression levels and functional assays.

C. Functional screening of breast cancer cell lines in response to TNF- α , Fas ligand and granzyme B/adenovirus.

According to the original plan, this part should have been done by the end of the second year. Because much effort was made to perform experiments presented in section A of this report, only part of this task has been done. The rest will be completed in a short time.

We performed functional screening of 11 cell lines in response to TNF- α (Fig. 4, Appendix A). When all these cell lines were treated with 80 ng/ml TNF- α plus 5 μ g/ml cycloheximide for four hours, each cell line displayed different response to the treatment. As determined by DAPI staining (it has better contrast between normal and apoptotic cells than Hoechst staining), it appeared that ZR-75-1, MDA-MB-436, MDA-MB-435S cell lines were prone to undergo apoptosis (these cells also had higher background). HS-578T and T47D are relatively resistant to TNF- α among the cells tested. In context with caspase expression, it is interesting to notice that MDA-MB-435s and MDA-MB-436

cells have higher levels of overall caspase expression. HS-578T has lower levels of overall caspase expression. Although our data can not attribute the survival rate of treated cell lines to a particular caspase, the connection between caspase expression and apoptotic response is emerging. Screening of more cell lines with Fas ligand and GrB/Ad is being carried out.

D. Analysis of chemotherapy and radiotherapy mediated killing in caspase aberrant cell lines.

This part of work was planned in months 23-29 of the project and we are a little ahead of original planing. We have performed MTT assay on 19 cell lines in response to doxorubicin treatment. Our original plan was to use selected cells lines with aberrant caspases. In view that the mechanism of drug treatment induced apoptosis may be different from TNF- α and Fas mediated apoptosis, we worked on more cell line so that the results would be a reference for the selection of cell lines for other drug treatments. As shown in Fig. 5-8 of Appendix A, MDA-MB-468, MDA-MB-436, MDA-MB-231, MDA-MB-157 were more susceptible to doxorubicin. HS-578T, which showed relatively resistant to TNF- α induced apoptosis, was sensitive to doxorubicin, suggesting that in addition to caspase expression, more factors might be involved in this process. In contrast, MDA-MB-415, BT-483 and MDA-MB-435S were less sensitive to doxorubicin. We would have a better summary on our findings after more results from these experiments are obtained.

Key Research Accomplishment

- We have characterized apoptosis in caspase 3 reconstituted MCF-7 cells treated with etoposide
- We have found that caspase 3 had strong feedback action on mitochondria in radiotherapy induced apoptosis and that cytoskeletal proteins are more susceptible to radiation induced apoptosis.

- We have established MCF-7/pv and MCF-7/c3 cells expressing dominant negative mutant p53 and performed preliminary study on interactions between p53 and caspase 3 in chemotherapy induced apoptosis.
- We have detected caspases 1, 2 and 4 in the nine cell lines reported last year, and detected caspases 1 – 10 (except caspase 5) in another nine breast cancer cell lines
- We have made total RNA from 19 breast cancer cell lines.
- We have performed functional screening on 11 breast cancer cell lines treated with TNF- α
- We have analyzed the sensitivity of 19 breast cancer cell lines in response to doxorubicin treatment

Reportable Outcomes

1. Publication/reprint: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin and etoposide induced apoptosis, Cancer Research, 61: 348-54, 2001 (Appendix D)
2. Manuscript: Radiation induced apoptosis in MCF-7 breast cancer cells reconstituted with caspase 3 (to be submitted) (Appendix B).
3. Thesis: The role of p53 in caspase 3 activation during chemotherapy induced apoptosis in MCF-7 breast cancer cell line (By Alison Johnson) (Appendix C)

Conclusion

Summary

In the second year of the project, our progress is reflected in the following aspects. First, we continued to examine caspase expression in breast cancer cell line. Together with last year's results, we have detected the expression of caspases 1 –10 (except caspase 5) in about 20 breast cancer cell lines. To determine the expression of other apoptotic regulators if necessary, we have made total RNA from 20 cell lines. Second, we performed functional screening of breast cancer cell lines treated with TNF- α and

doxorubicin. We have analyzed apoptosis of 11 TNF- α treated cell lines using DAPI staining and 19 doxorubicin treated cell lines using MTT assay. The data up to date suggested that one cell line that is sensitive to TNF- α might response to doxorubicin differently, given their different apoptotic mechanism. Detailed conclusion needs more experiments. The third, we continued our investigation on the specific role in chemotherapy and radiotherapy induced apoptosis. Our results indicated that caspase 3 plays a critical role in chemotherapy and radiotherapy induced apoptosis and suggest that caspase 3 deficiency might contribute therapeutic resistance. Some of the data has been published in Cancer Research.

“So what section”

The experiments in our proposal could be divided into two major parts. One is to identify low/deficient caspase expression and the other is to study the correlation between caspase expression and carcinogenesis/resistance. Our results have not found a complete caspase deficient cell line in the scope we researched. But we did see the expression of lower levels of certain caspases. We still believe that lower level/deficient expression of certain caspase may contribute to cancer carcinogenesis, as supported by recent reports showing that more caspase deficiency was identified in other tumors (1, 2). Since apoptotic regulation is a complicated net work, which may be due to functional redundancy, we have to be prepared to analyze the data in an “array style” to generate closer conclusion. We also realized that tissue explant experiments were not practical, given the slow growth of epithelia cells and fibroblast contamination. For the purpose to detect caspase expression in breast cancer, immunohistochemistry detection on tumor specimen would be a good alternative in the following years. In context with caspase expression data, detecting the expression of other apoptotic regulators, such as Bcl-2 family members and death receptors, would be helpful in explaining the functional screening data.

Our caspase 3 deficient/reconstituted MCF-7 cell line model provided us excellent opportunity to study the specific role of caspase 3. While identifying more caspase

aberrant breast cancer cell line and generating new hypothesis from screening data, we will make further effort in studying the role caspase 3 in tumor biology.

References

1. Wrone-Smith, T., Izban, K. F., Ergin, M., Cosar, E. F., Hsi, E. D., and Alkan, S. Transfection of caspase-3 in the caspase-3-deficient Hodgkin's disease cell line, KMH2, results in enhanced sensitivity to CD95-, TRAIL-, and ARA-C-induced apoptosis, *Exp Hematol.* 29: 572-81., 2001.
2. Schwartz, S., Jr., Yamamoto, H., Navarro, M., Maestro, M., Reventos, J., and Perucho, M. Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype, *Cancer Res.* 59: 2995-3002, 1999.

Appendices

- A: Data from the detection of caspase expression and functional screening in breast cancer cell lines (8 figures)
- B: Manuscript: Radiation induced apoptosis in MCF-7 breast cancer cells reconstituted with caspase 3
- C: Thesis: The role of p53 in caspase 3 activation during chemotherapy induced apoptosis in MCF-7 breast cancer cell line (By Alison Johnson)
- D: Reprint: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin and etoposide induced apoptosis, *Cancer Research*, 61: 348-54, 2001

Appendix A

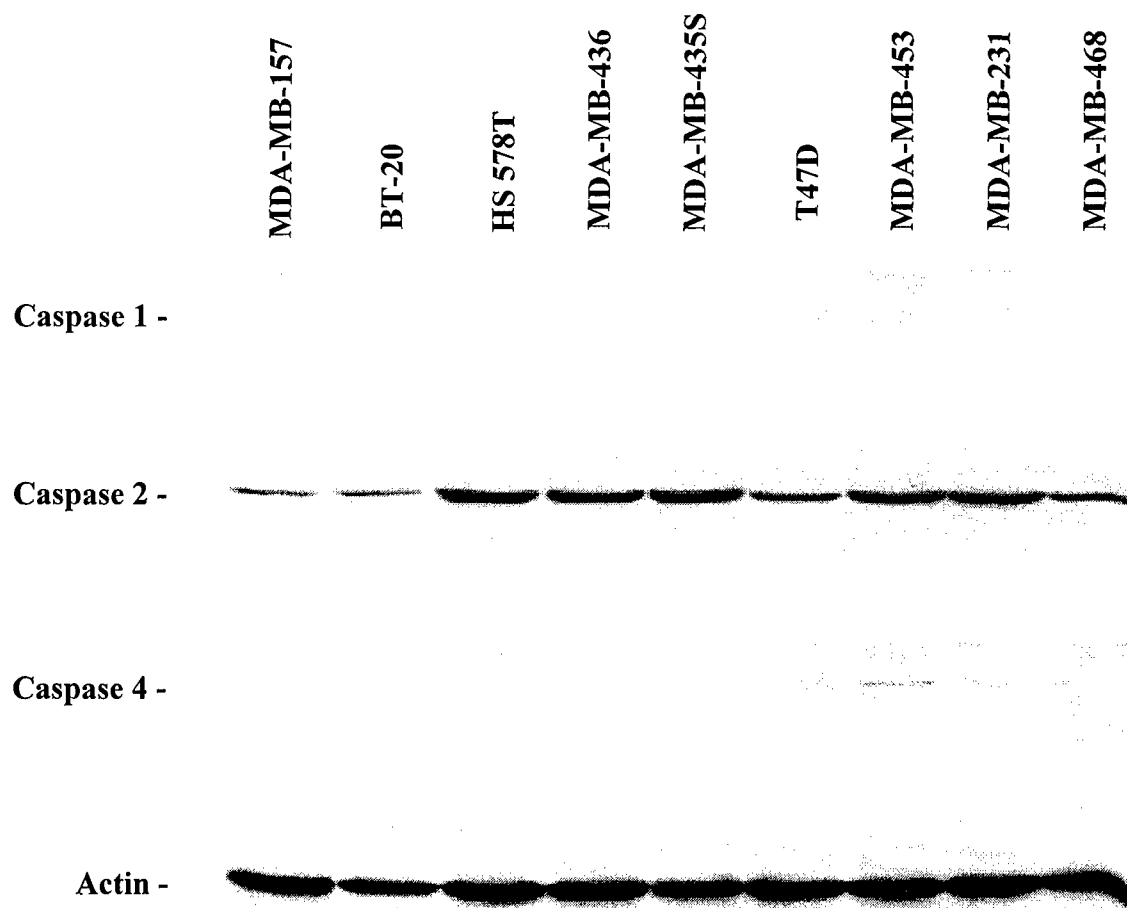


Fig. 1. Protein levels of caspases 1, 2 and 4 in 9 breast cancer cell lines

Protein lysate was made from indicated breast cancer cell lines. Individual caspases were probed with corresponding specific antibodies using Western Blotting. Actin levels are shown as loading control

Appendix A

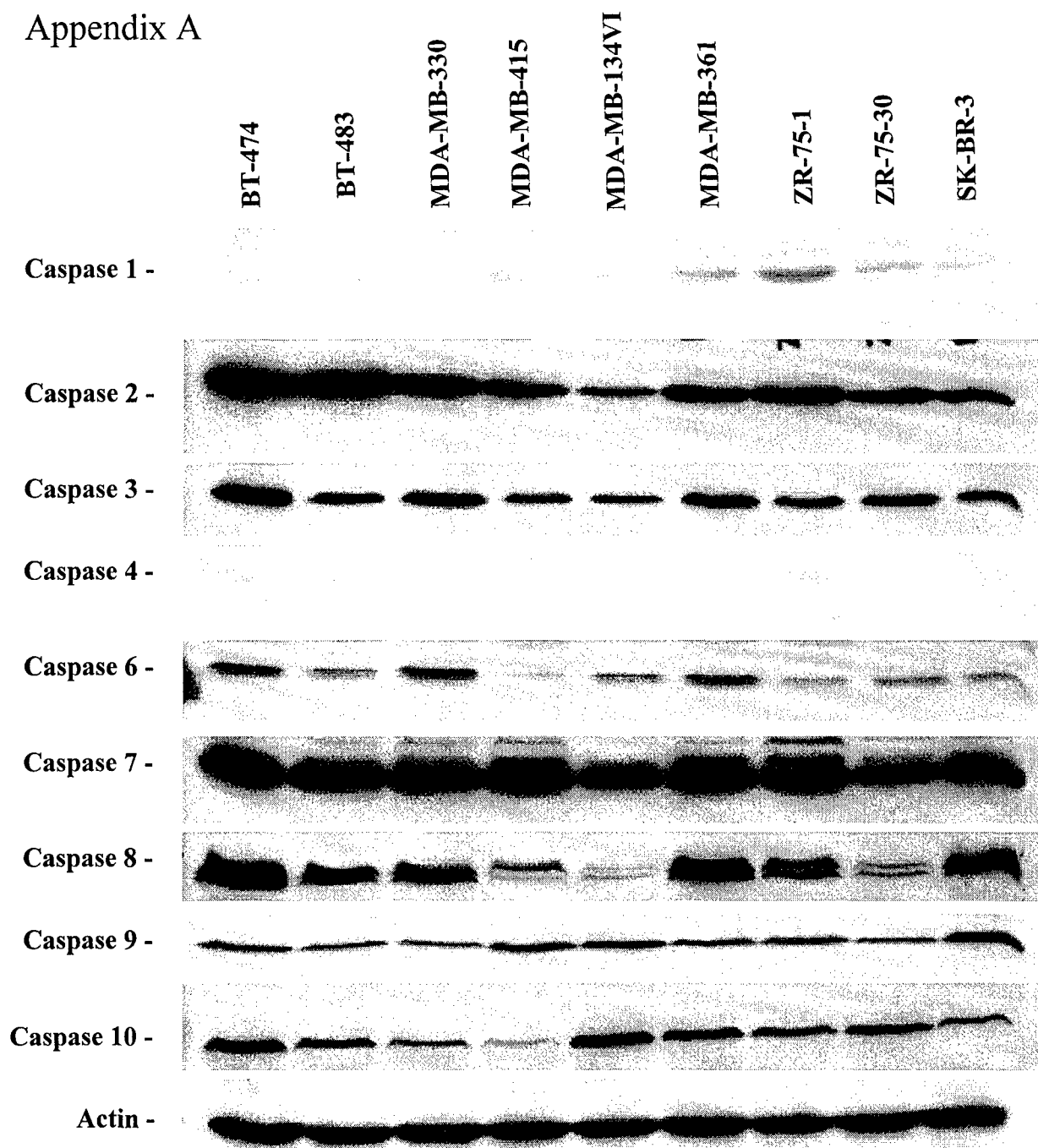


Fig. 2. Protein levels of caspases 1, 2, 3, 4, 6, 7, 8, 9 and 10 in 9 breast cancer cell lines

Protein lysate was made from indicated breast cancer cell lines. Individual caspases were probed with corresponding specific antibodies using Western Blotting. Actin levels are shown as loading control

Appendix A

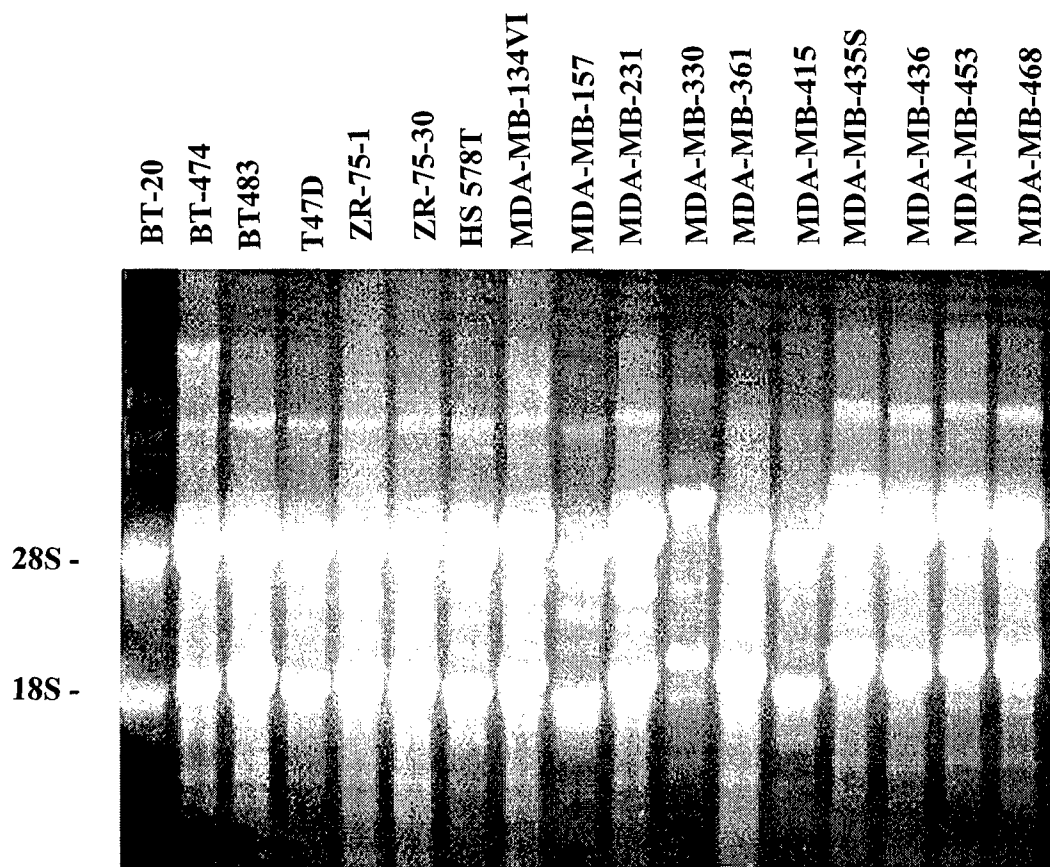


Fig. 3. RNA extraction from 17 breast cancer cell lines

Total RNA was extracted from exponential growing cells using
Trazol reagent

Appendix A

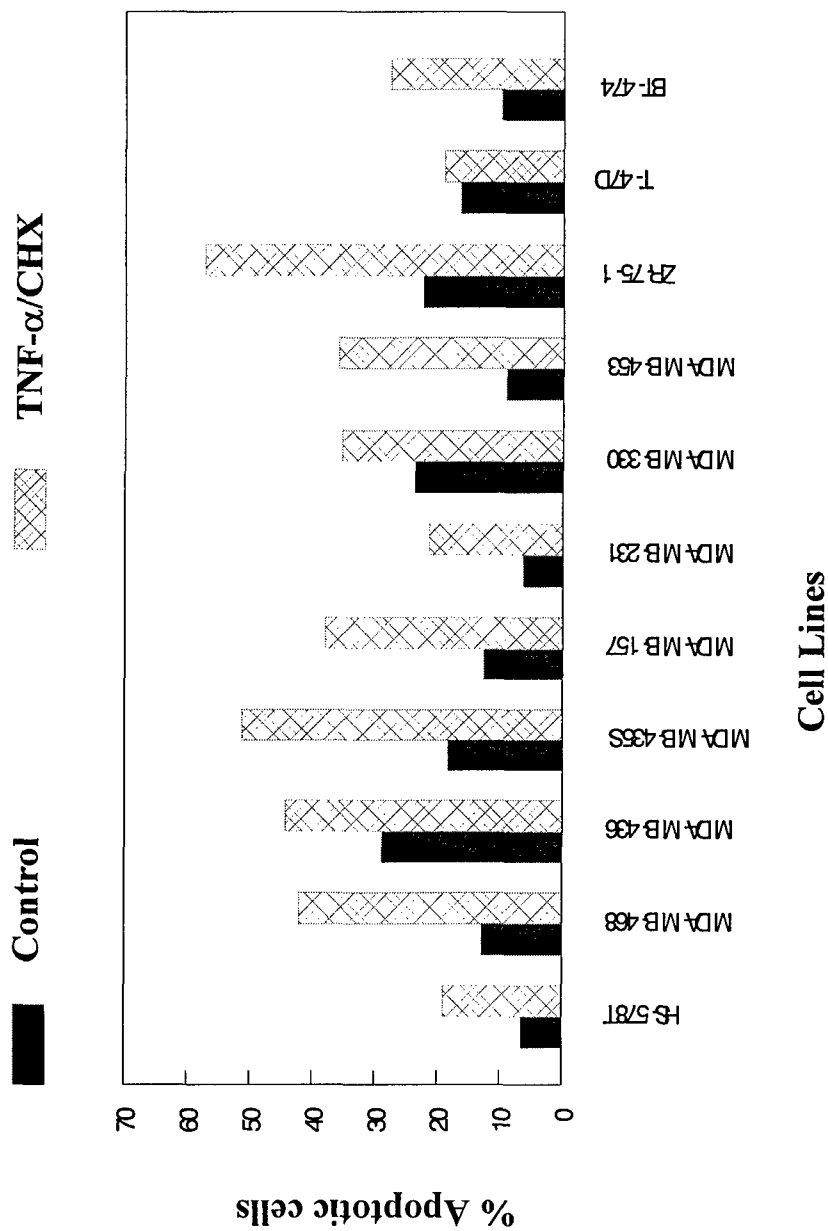


Fig. 4. Apoptosis in breast cancer cell lines treated with TNF- α

The cells were treated with medium alone (control) and 80 ng/ml TNF- α plus 5 μ g/ml cycloheximide for 4 hrs. Apoptosis was analyzed using DAPI staining

Appendix A

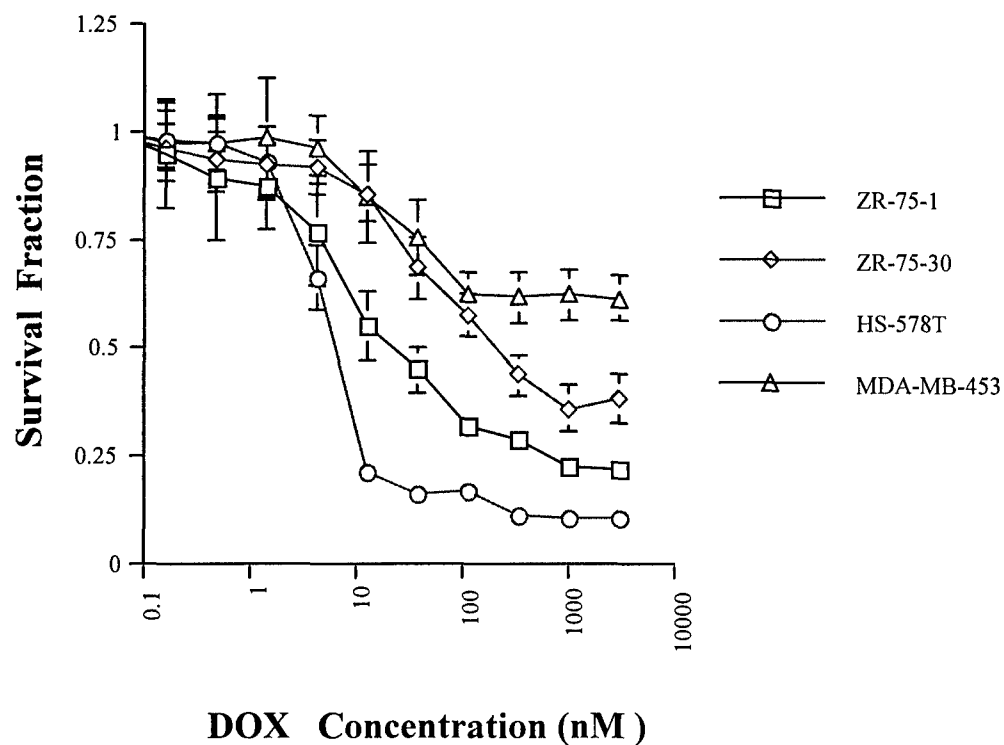


Fig. 5. Survival fractions of ZR-75-1, ZR-75-30, HS-578T and MDA-MB-453 breast cancer cells in response to doxorubicin.

Different cell lines were treated with doxorubicin for 6 days before the survival fractions were determined using MTT assay. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix A

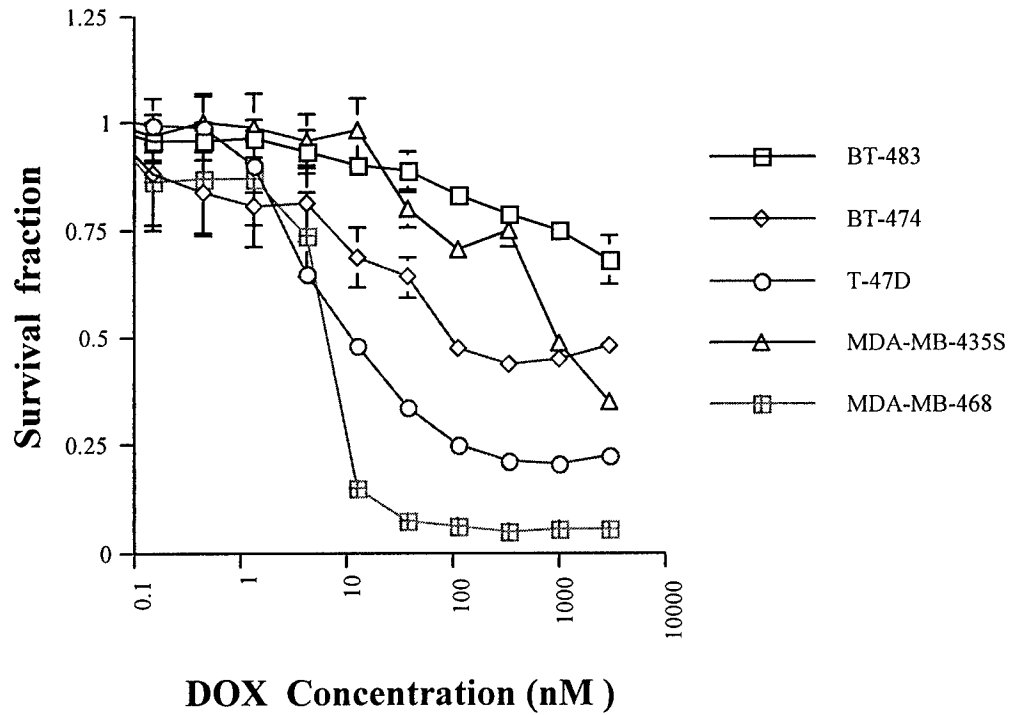


Fig. 6. Survival fractions of BT-483, BT-474, T-47D, MDA-MB-435S and MDA-MB-468 breast cancer cells in response to doxorubicin. Different cell lines were treated with doxorubicin for 6 days before the survival fractions were determined using MTT assay. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix A

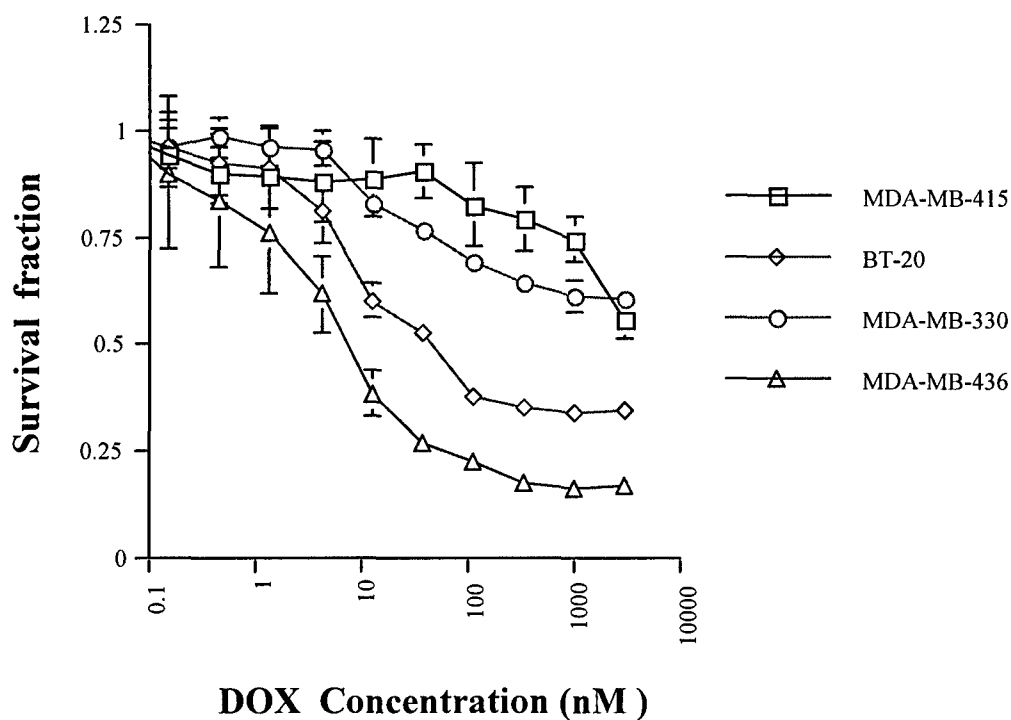


Fig. 7. Survival fractions of MDA-MB-415, BT-20, MDA-MB-330, MDA-MB-436 breast cancer cells in response to doxorubicin.

Different cell lines were treated with doxorubicin for 6 days before the survival fractions were determined using MTT assay. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix A

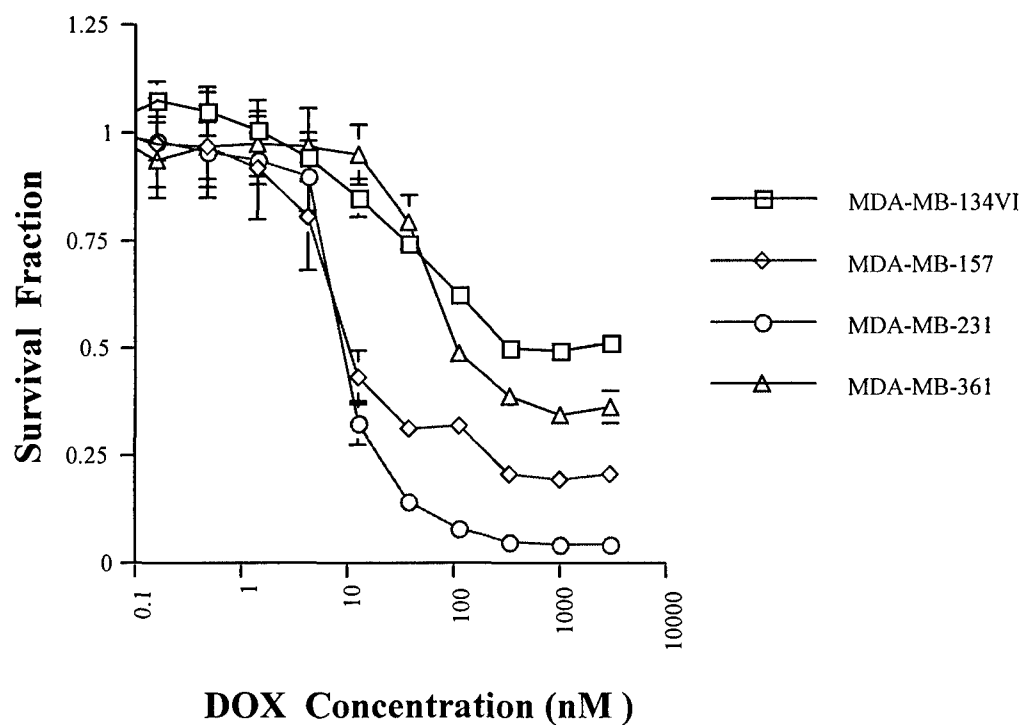


Fig. 8. Survival fractions of MDA-MB-134VI, MDA-MB-157 , MDA-MB-231, MDA-MB-361 breast cancer cells in response to doxorubicin. Different cell lines were treated with doxorubicin for 6 days before the survival fractions were determined using MTT assay. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix B

Radiation Induced Apoptosis in MCF-7 Breast Cancer Cells Reconstituted with Caspase 3¹

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Running title: Caspase 3 reconstitution mediated sensitization to radiotherapy

ABSTRACT

To study the specific role of caspase3 in radiotherapy induced apoptosis, we investigated the apoptotic activities of caspase 3 deficient MCF-7 (MCF-7/pv) cells and MCF-7 cells reconstituted with caspase 3 (MCF-7/c3) in response to ionizing radiation. MTT assays indicated that caspase 3 reconstitution significantly enhanced radiation mediated killing in MCF-7/c3 cells. Increased caspases 6 and 7 activation was detected in irradiated MCF-7/c3 cells, in which caspase 7 activation was caspase 3 dependent. As a result, the cleavage of cellular death substrates PARP, lamin B and actin was remarkably amplified. Examination of mitochondrial depolarization of both cell lines suggests that caspase 3 had a strong feedback action on mitochondria. Comparison of death substrate cleavage among apoptosis induced by radiation, doxorubicin and TNF- α treatment revealed that cytoskeletal proteins might be more susceptible in ionizing radiation induced apoptosis. Taken together, our results demonstrated the critical role of caspase 3 in radiotherapy induced apoptosis, and suggests that caspase 3 deficiency might contribute to radioresistance.

Key words: Caspase 3; Apoptosis; Radiotherapy; Lamin B and Actin

INTRODUCTION

Accumulated studies have indicated that apoptosis is involved in different anti-tumor therapies, including radiotherapy (1). Effective tumor killing in these therapies requires intact apoptotic machinery. According to the sequential events during apoptosis, apoptotic process could be divided into initiation and execution stages (2). Although numerous stimuli could trigger apoptosis, different apoptosis share a relatively common execution process, which is the activation of caspase cascade (3). Depending on the type of the stimuli, the initiation process may vary to a certain extent. However, apoptosis induced by most stimuli is initiated through one of the two major pathways, the death receptor pathway and mitochondrial pathway (2).

Despite the detailed mechanism of radiation induced apoptosis remain unclear, increasing reports suggest that radiation induced apoptosis is predominantly through mitochondrial pathway (1). Radiation mediated DNA damage induce p53 expression, which in turn regulate the transcription of other apoptotic regulators, such as Bax (4). Alteration of dimerization among bax and Bcl-2 family members and activation of caspase 8 may lead to mitochondrial membrane permeability changes and release of cytochrome c (5-7). Complex formation among cytochrome c, caspase 9 and apaf-1 induces caspase 9 activation, which is followed by the activation of effector caspases. In addition, ceramide production and upregulation of death receptors were also reported to be involved in radiation induced apoptosis (8, 9).

Aberrant apoptosis has been associated with resistance to radiotherapy. A radio-resistant variant derived from human neuroblastoma cell lines was found to be less prone to radiation induced apoptosis (10). Loss of p53 function is correlated to apoptotic defect

and radiation resistance (11). Resistant to radiation induced apoptosis in a lymphoma cell line was associated with defect ceramide signaling . Overexpression of Bax, which is an apoptotic promoting factor of Bcl-2 family and is down regulated in MCF-7 cells, sensitized those cells to radiation mediated apoptosis (12). In this context, we investigated the possible contribution of caspases, caspase 3 in particular, deficiency to radiation resistance.

Caspases are a group of special cysteine proteases, which could be classified into two groups (3, 13). Apical caspases, such as caspases 2, 8, 9 and 10 are involved in apoptosis initiation. Effector caspases, such as caspases 3, 6 and 7, are responsible for the cleavage of cellular substrates, which results in cellular dysfunction and disassembly. Among them, caspase 3 is one of the key caspases in apoptosis execution (14). As a major target of apical caspases, activated caspase 3 cleave a number of cellular death substrates, such as poly ADP-ribose polymerase (PARP), DNA fragmentation factor (DFF), protein kinase c and gelsolin (3). Caspase 3 activity has been detected in the apoptosis induced by various stimuli. However, caspase 3 knockout mice showed abnormal apoptosis in selected tissues, but not all tissues (15). MCF-7 cells, which are deficient of caspase 3, can eventually undergo apoptosis (16, 17). This dilemma attracts further study on the specific role of caspase 3 in different apoptosis.

In stead of using synthetic caspase 3 inhibitors (18, 19), such as DEVD-CHO, which are not specific enough (20), we studied the specific role of caspase 3 by comparing apoptotic characteristics between caspase 3 deficient MCF-7 (MCF-7/pv) and MCF-7 cells reconstituted with caspase 3 (MCF-7/c3). In our previous report, we found that reconstitution of caspase 3 sensitized MCF-7 cells to chemotherapeutic agents like

doxorubicin and etoposide (21). Although the mechanism of radiation induced apoptosis is generally believed to be similar to chemotherapy induced apoptosis, given the complexity of apoptosis regulation, there might be detailed variation in the execution of radiation induced apoptosis. Therefore, while investigating the correlation between caspase 3 deficiency and radioresistance, we also examined possible difference among chemotherapy and TNF- α induced apoptosis. We found that caspase 3 reconstitution also significantly enhanced radiation induced apoptosis. The enhancement was mediated by increased activity of caspases 3, 6 and 7 in irradiated MCF-7/c3 cells. Feedback action of caspase 3 on mitochondria may also contribute to this process. In addition, Our results suggested that cleavage of actin and lamin B, which are cytoskeleton and nuclear matrix proteins, is more susceptible in ionizing radiation induced apoptosis.

MATERIALS AND METHODS

Cell lines and cell culture

MCF-7/pv and MCF-7/c3 cells were established as described before (21). MCF-7/pv cells were MCF-7 cells transfected with control pBabepuro vector. MCF-7/c3 cells were MCF-7 cells reconstituted with caspase 3 using a retroviral system. All cells were maintained in IMDM medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

Radiation and drug treatment

The cells were irradiated using Varian Clinac 4 photon Linear. For MTT assay, cell suspension was irradiated at appropriate dosages and then inoculated into 96 well plates. For the other assays, cells growing in 60-mm tissue culture dishes at 80% confluence were irradiated or treated with 10 μ M doxorubicin or 80 ng/ml TNF- α and 5ug/ml cycloheximide (CHX) at indicated dosage or duration.

MTT assay

After the cells were irradiated in cell suspension, 500 cells of each sample were inoculated in 96 well plates. Six days after treatment, the medium was changed into phenol red free medium containing 500ug/ml MTT. 3 hours after incubation, MTT containing medium was aspirated out. The incorporated dye was dissolved in 100 μ l/well of DMSO. The plates were read at 560/620 using an Elisa reader. Eight parallel samples were treated in each concentration point. Three separate experiments were performed. IC₅₀ values were determined by interpolation of the plotted data to show the drug concentration that produced 50% growth inhibition as compared to the control.

DEVD cleavage

The treated cells were washed with PBS and resuspended in lysis buffer (50 mM pH8.0 Tris-HCl, 130 mM KCl, 1mM EDTA, 10 mM EGTA and 10 μ M digitonin) at 10⁷ cells/ml). Following incubation at 37 C for 10 Min, the samples were spin for 3 Min (5000 rpm) and the supernatant was collected. Add 100 μ l of the lysate to each well of fluorometer plate. Add 100 μ l of substrate solution (2 μ M DEVD-AMC in lysis buffer) right before fluorometer reading. Repeat measurement every 10 min up to 60 min. T60-T0 = Δ mOD change over defined time.

Western blot

PBS washed cells were lysed by addition of lysis buffer and sitting on ice for 30 min. Lysed cells were centrifuged at 14,000 rpm for 10 min to remove cell debris. Protein in the supernatant was determined using BCA protein Assay (Pierce, Rockford, IL). 50 μ g of cell lysate was loaded on each lane. Protein was separated by either 10% or 15% SDS-PAGE gels and transferred to nitrocellulose membrane. The membrane was blocked with 5% milk in TBS-T washing buffer. The concentration of the primary antibodies was used at 1:500 to 1:2000 dilution. Washed membrane was then probed with HRP labeled secondary antibodies. The membrane was washed again and detected with ECL reagent (Amersham-Pharmacia, IL) and the specific protein was visualized by autoradiography.

Flow cytometry

Twenty-four hours after irradiation, the cells were trypsinized and washed with PBS. For DNA content analysis, the cells were then fixed in 50 μ l of 0.125% paraformaldehyde in PBS at 37° C for 5 minutes, followed by the addition of 450 μ l of ice-cold methanol to each sample. After being washed three times with PBS containing 0.1% Triton X-100 and treated with RNase A (0.04 Kunitz units) for 30 minutes, the cells were then stained with 50 μ g/ml of propidium iodide. Cell analysis was performed using a Coulter Epics 751 flow cytometer. The fraction of the total cell population present in each of G1, S, G2/M and the hypodiploid peak was obtained from DNA histograms by mathematical modeling using MPLUS software. For mitochondrial depolarization analysis. Trypsinized cells were stained with 10 μ g/ml JC-1 (Molecular Probe Inc.) for 10 min and analyzed using flow cytometry immediately.

RESULTS

1. Enhanced killing in caspases 3 reconstituted MCF-7 cells in response to radiation

MTT assays were performed to examine the sensitivity of MCF-7/pv and MCF-7/c3) in response to radiation. Six days after the cells were irradiated at dosages from 2.5 to 15 Gy, the survival fractions of MCF-7/c3 cells were consistently lower than that of MCF-7/pv cells at any dosage (Fig. 1). Statistical analysis indicated that the ID₅₀ of irradiated MCF-7/c3 cells was 4.20 Gy, which was significantly ($p < 0.01$) lower than that of MCF-7/pv cells (6.3 Gy) (Table 1). The result indicated that reconstitution of caspase 3 in MCF-7/c3 cells rendered the cells more sensitive to radiation mediated killing.

2. Activation of caspases 3, 6 and 7 in irradiation MCF-7/c3 cells

To confirm that enhanced killing in irradiated MCF-7/c3 cells was because of caspase 3 reconstitution, we detected caspase 3 like activity in control and MCF-7/c3 cells using DEVD cleavage assay. As shown in Fig. 2, 24 hrs after irradiation, caspase 3 like activities were at basal levels in all groups of MCF-7/pv cells, even in the cells irradiated with 50 Gy. However, caspase 3 like activity increased significantly in irradiated MCF-7/c3 cells, suggesting that activation of reconstituted caspase 3 might contribute to the enhanced killing in MCF-7/c3 cells.

Activation of caspases 6 and 7 in irradiated MCF-7/pv and MCF-7/c3 cells were analyzed using western blotting. Twenty four hrs after MCF-7/pv cells were irradiated at 100 to 180 Gy dosages, caspases 6 was activated at very low levels, as indicated by the

appearance of cleaved sub-units (Fig. 3), suggesting that caspase 3 activity was not necessary for caspase 6 activation. However, caspase 7 activation was not detectable in MCF-7/pv cells. In contrast, activation of caspases 6 and 7 was remarkably increased/detected in irradiated MCF-7/c3 cells. These results, which showed that caspase 7 activation was dependent of caspase 3 and that caspase 6 activation would be significantly enhanced in the presence of caspase 3, were consistent with our finding in chemotherapy and granzyme B induced apoptosis.

3. Cleavage of death substrates PARP, lamin B and actin in MCF-7/pv and MCF-7/c3 cells

To measure the consequence and extent of caspase activation in irradiated MCF-7/pv and MCF-7/c3 cells, we detected the cleavage of cellular death substrates PARP, lamin B and actin in both cell lines. In irradiated MCF-7/pv cells, the cleavage of PARP, lamin B and actin was at low levels (Fig. 4A, 4B and 4C). In contrast, with reconstitution of caspase 3, cleavage of these substrates in irradiated MCF-7/c3 cells increased significantly. This was consistent with the results from doxorubicin and TNF- α treated cells. The results indicated that caspase 3 activity is critical for acquiring maximal destruction in irradiated cells.

To find any clue that may lead to unique feature in radiation induced apoptosis, we compared the cleavage of these substrates in irradiated cells with that in the cells treated with doxorubicin and TNF- α . The comparison revealed that, under the given conditions, there might be a preference of certain subset of substrates for different apoptosis.

Compared to that in doxorubicin and TNF- α treated cells, PARP cleavage in irradiated MCF-7/pv and MCF-7/c3 cells was not prominent (Fig 4A), even though it increased remarkably in the presence of caspase 3. However, PARP was most completely cleaved in MCF-7/c3 cells treated with 10 μ M doxorubicin for 12-20 hrs or TNF- α for 4-6 hrs.

When the cleavage of lamin B was compared among the three treatments, it appeared that lamin B was more susceptible to radiation. In irradiated MCF-7/pv cells, little or no lamin B cleavage was detected in doxorubicin and TNF- α treated cells (Fig. 4B). However, it was readily detected even in irradiated MCF-7/pv cells and more in MCF-7/c3 cells. In addition, while no cleavage of actin was detected in both cells lines treated with either doxorubicin or TNF- α under the given condition (Fig. 4C), actin cleavage was detected in irradiated MCF-7/pv and especially in MCF-7/c3 cells. These results suggest that cleavage of lamin B and actin was not caspase 3 dependent. With caspase 3 activity, however, their cleavage increased significantly.

Since it was reported that ionizing radiation induced accumulation of cytoskeletal elements when the cells were blocked in G2/M phases of cell cycle (22), we analyzed the cell cycle status of irradiated MCF-7/pv and MCF-7/c3 cells. 24 hours after the cells were irradiated at 50 Gy, the number of the cells in G2/M phases increased significantly in both cell lines (Fig. 5). However, establishment of susceptibility of cytoskeletal elements and cell cycle blocking requires further studies.

4. Caspase 3 has feedback action on mitochondria in irradiated MCF-7/c3 cells

Mitochondria plays a central role in apoptosis induced by intracellular signals, such as DNA damage (9). It has been demonstrated that caspase 3 works downstream of mitochondria, which releases cytochrome c in response to cellular stresses. Since recent reports suggested that caspase 3 could act on some apical factors, such as Bid and Bcl-2 (23, 24), we looked at the possible feedback action of caspase 3 on mitochondria by detecting mitochondrial depolarization using JC-1 staining and flow cytometry. JC-1 is a double-color fluorescent dye. Non-depolarized mitochondria are usually stained with more red and less green. Depolarized mitochondria would display increased green and decreased red. As shown in Fig. 6, cells in upper right (UR) and lower right (LR) Quadra represent the cells with depolarized mitochondria. In MCF-7/pv cells irradiated at 50 Gy, mitochondria depolarization was moderate, which were about 13% as compared to 3% in unirradiated control cells. In contrast, about 33% in irradiated MCF-7/C3 cells had depolarized mitochondria. Increased depolarization in irradiated MCF-7/c3 cells suggests that feedback action of caspase 3 might contribute to the enhanced apoptosis in irradiated MCF-7/C3 cells.

DISCUSSION

To study the role of caspase 3 in radiation induced apoptosis in breast cancer, we characterized the apoptotic activities in irradiated caspase 3 deficient (MCF-7/pv) and reconstituted (MCF-7/c3) cells. Since we have found that caspase 3 reconstitution sensitized MCF-7 cells to chemotherapeutic agent induced apoptosis, it would be speculated that caspase 3 reconstitution could also sensitize these cells to radiation

induced apoptosis. However, some groups reported that inhibition of caspase 3 using synthetic inhibitors did not contribute survival changes (25). Given the redundancy of caspase action and the complexity of apoptotic regulation, using our caspase 3 specific cell line model would provide direct evidence showing the functional role of caspase 3 in ionizing radiation induced apoptosis.

Our results from MTT experiments showed that caspase 3 reconstitution indeed sensitized MCF-7 cells to radiation mediated killing, as indicated by consistently lowered survival fraction and ID50 in MCF-7/c3 cells. Activation of caspase 3, enhanced activation of caspases 6 and 7 and amplified cleavage of cellular death substrates, such as PARP, lamin B and actin in MCF-7/c3 cells, demonstrated increased apoptotic activities. More mitochondrial depolarization in irradiated MCF-7/c3 cells suggests that feedback action of caspase 3 also contributed to the sensitization process. These results bridged the gap between caspase 3 reconstitution and the increased killing efficacy in irradiated MCF-7/c3 cells, suggesting that functional caspase 3 is required for efficient radiotherapy and that caspase 3 deficiency might contribute radioresistance. Since caspase 3 deficiency has been detected in other tumor cells (26) and in breast cancer specimens (unpublished data), correlation between caspase 3 levels and function and therapeutic resistance would have some impact on clinical oncology.

Another interesting finding of these experiments is that it appears that cytoskeletal proteins are more susceptible to be cleaved in radiation induced apoptosis. Since lamin B and actin were cleaved in both MCF-7/pv cells, it suggests that it was not caspase 3

dependent. In fact, it was reported that lamin proteins were preferred substrates of caspase 6 (27). However, reconstitution of caspase 3 significantly enhanced the cleavage of the two cytoskeletal proteins. This might be the result of direct action of caspase 3 on these proteins, though increased caspases 6 activity or both. With the same biochemical background of the cells treated with TNF- α and doxorubicin, lamin B and actin cleavage was more susceptible to radiation suggested that other factors might be involved in radiation induced apoptosis. In another words, although increasing number of cellular death substrates are being identified, they are not equally susceptible in different apoptosis. There might be a preferred subset of substrates for apoptosis induced by certain stimuli.

Radiation dosage used in our study varied in deferent experiments. In MTT experiments, the cells were irradiated at 2.5 to 15 Gy and the survival fractions were measured 6 days after irradiation. Much higher dosages were used in caspase and death substrates cleavage analysis for several reasons. First, MCF-7 cells were resistant to radiation in nature, as shown by little cleavage of caspase and death substrates in MCF-7/pv cells. Second, radiation induced apoptosis takes a longer process. Unlike fractionated radiation used in clinical, we treated the cells with one time irradiation and collected the sample 24 hrs later. High doses were used to detect credible cleaved bands. Although the high does are not applicable in clinical, the reaction pattern of apoptotic activities would be expected to be proportional.

In summary, our experiments demonstrated that reconstitution of caspase 3 sensitized MCF-7 breast cancer cells to radiotherapy induced apoptosis. This process was mediated by increased activation of caspases 6 and 7, amplified cleavage of cellular death substrates and the feedback action of caspase 3 on mitochondria, suggesting the critical role of caspase 3 in radiation induced apoptosis. Our results also suggest that cytoskeletal proteins might be more susceptible in ionizing radiation induced apoptosis.

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LEGENDS

Fig. 1. Caspase 3 reconstitution sensitizes MCF-7 cells to ionizing radiation induced apoptosis. MCF-7/pv and MCF-7/c3 cells were inoculated in 96 well plates after irradiation. Survival fractions were detected 6 days later using MTT assay.

Fig. 2. DEVD cleavage activity in irradiated MCF-7/pv and MCF-7/c3 cells. The cells were irradiated at indicated doses 24 hours before the lysate was prepared for fluorogenic assay.

Fig. 3. Western blot showing the activation of caspases 6 and 7 irradiated MCF-7/pv and MCF-7/c3 cells. The cells were irradiated at the indicated dose 24 hours before the lysate was prepared for Western blot. Fifty μ g of lysate protein was separated with SDS-PAGE gel. The caspases were probed with specific antibodies against caspase 6 and 7, respectively.

Fig. 4. Proteolytic cleavage of PARP, lamin B and actin in irradiated, doxorubicin and TNF- α treated MCF-7/pv and MCF-7/c3 cells. The conditions cell irradiation were the same as in Fig. 3. Both cell lines were also treated with 10 μ M doxorubicin or 80 ng/ml TNF- α plus 5 μ g/ml CHX for indicated duration. Cleavage of PARP (A), lamin B (B) and actin (C) was detected with corresponding specific antibody, respectively.

Fig. 5. Cell cycle analysis of irradiated MCF-7/ and MCF-7/c3 cells. The cells were collected, fixed and stained 24 hours after irradiation. DNA content of the treated cells was detected using flow cytometry.

Fig. 6. Mitochondrial depolarization in irradiated MCF-7/pv and MCF-7/c3 cells. Control and irradiated cells were collected 24 hours after irradiation. The cells were stained with JC-1 and analyzed using flow cytometry immediately after collection.

Table 1. Effects of ionizing radiation on survival fractions of MCF-7/pv and MCF-7/c3 cells

Treatment	MCF-7/pv		MCF-7/c3		p value
	ID ₅₀ ^a	95% CI ^b	ID ₅₀	95% CI	
Dosage (Gy)	6.30	5.87 - 6.73	4.02	3.79 - 4.25	< 0.01

^a Mean of ID₅₀s. Three data sets were used for analysis

^b Confidence Intervals

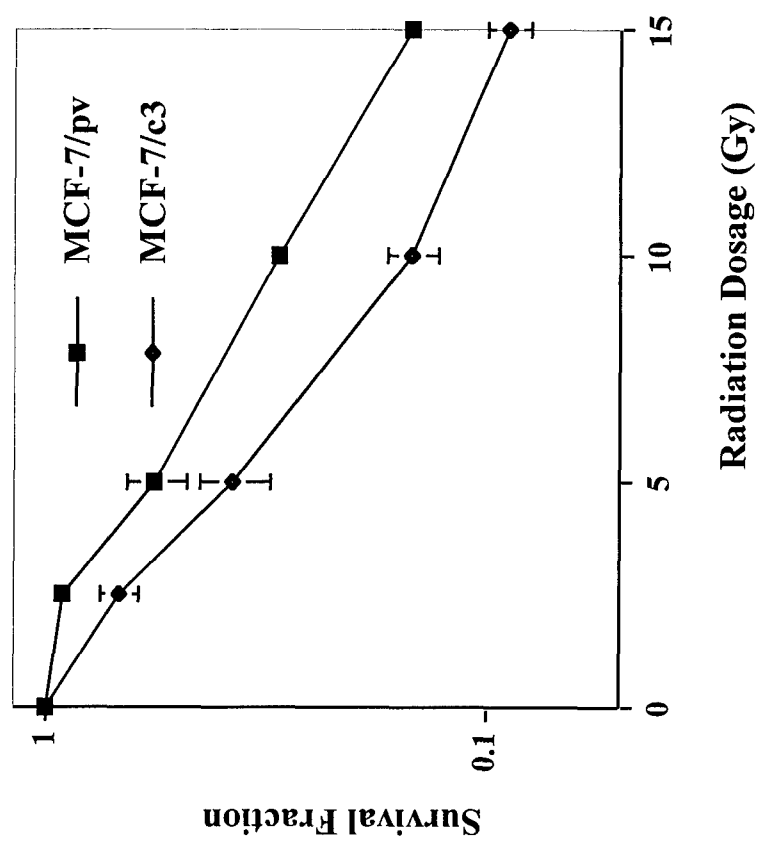


Figure 1

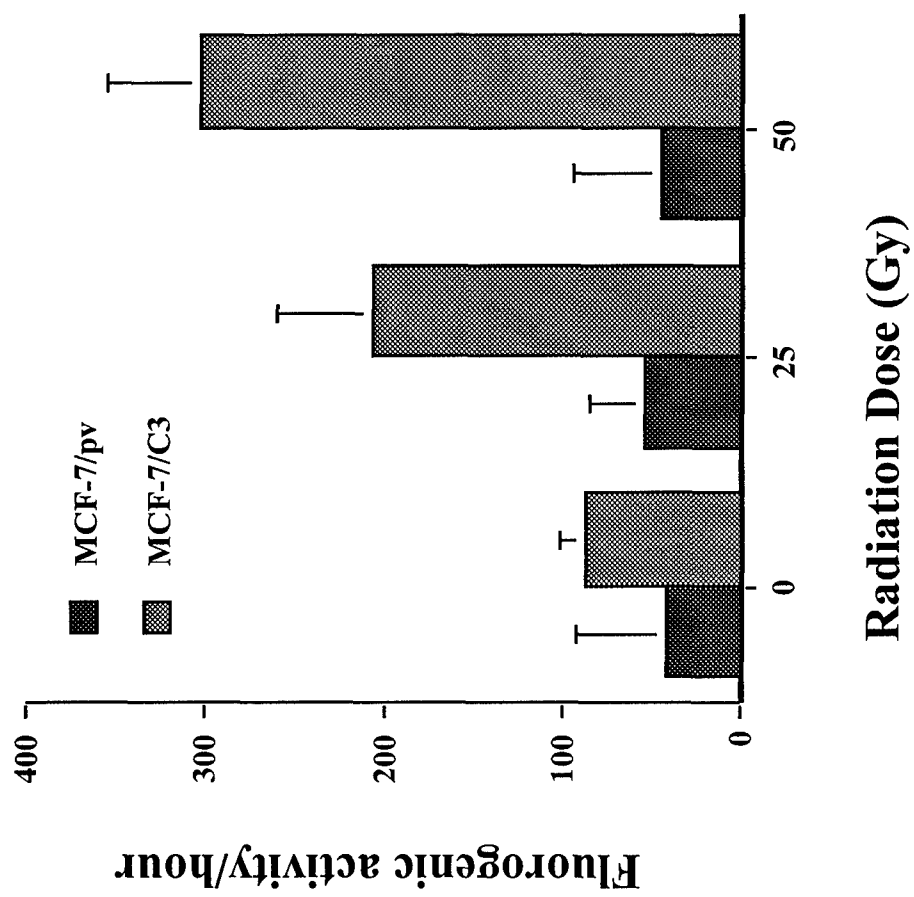


Figure 2

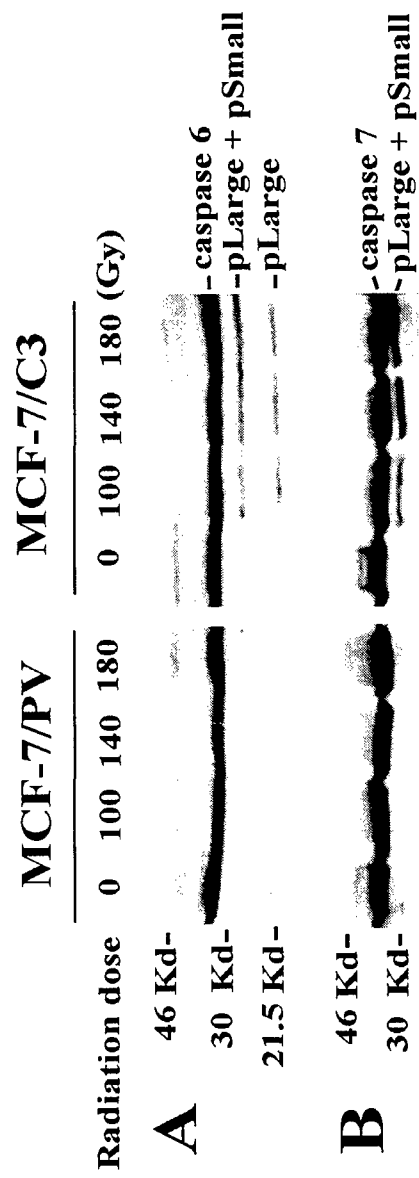


Figure 3

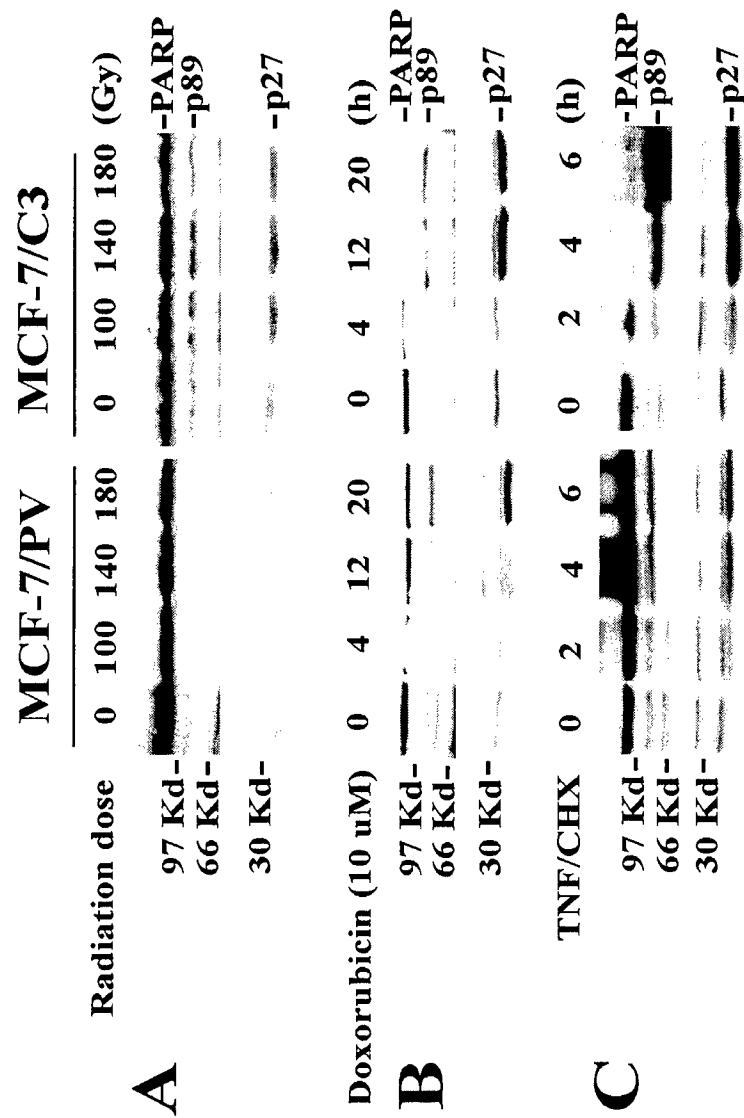


Figure 4A

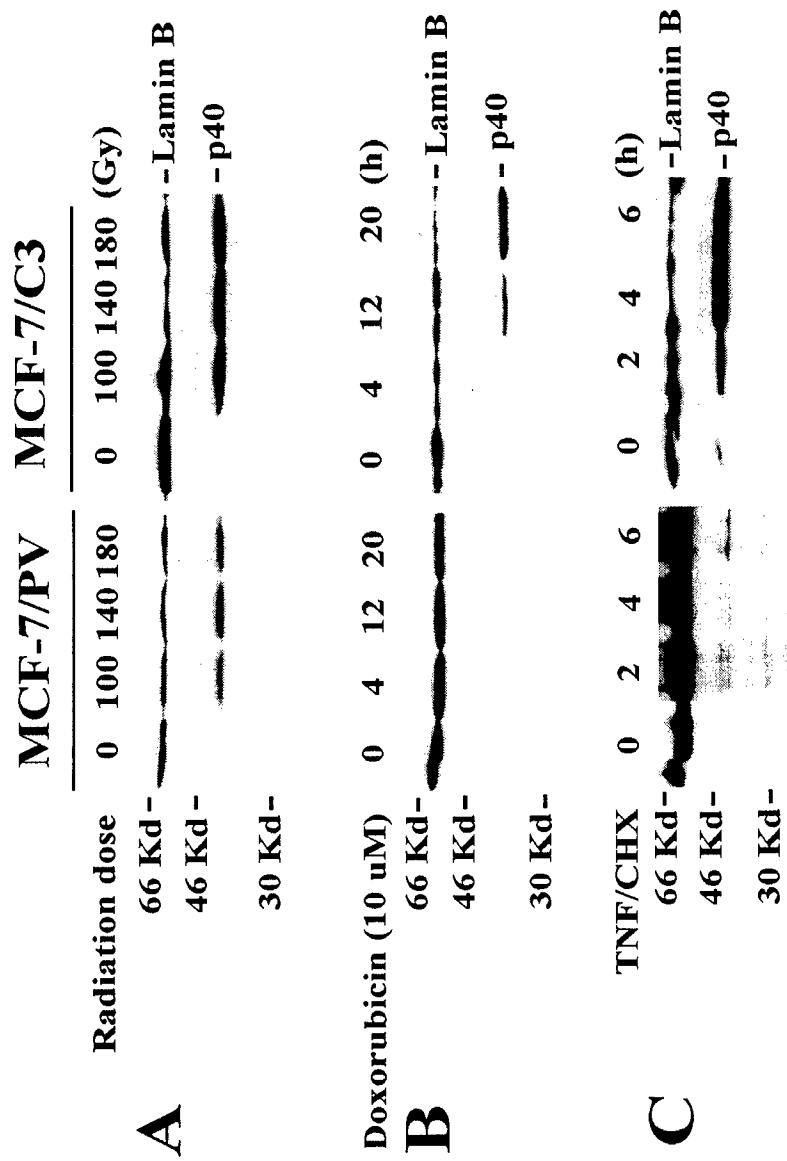


Figure 4B

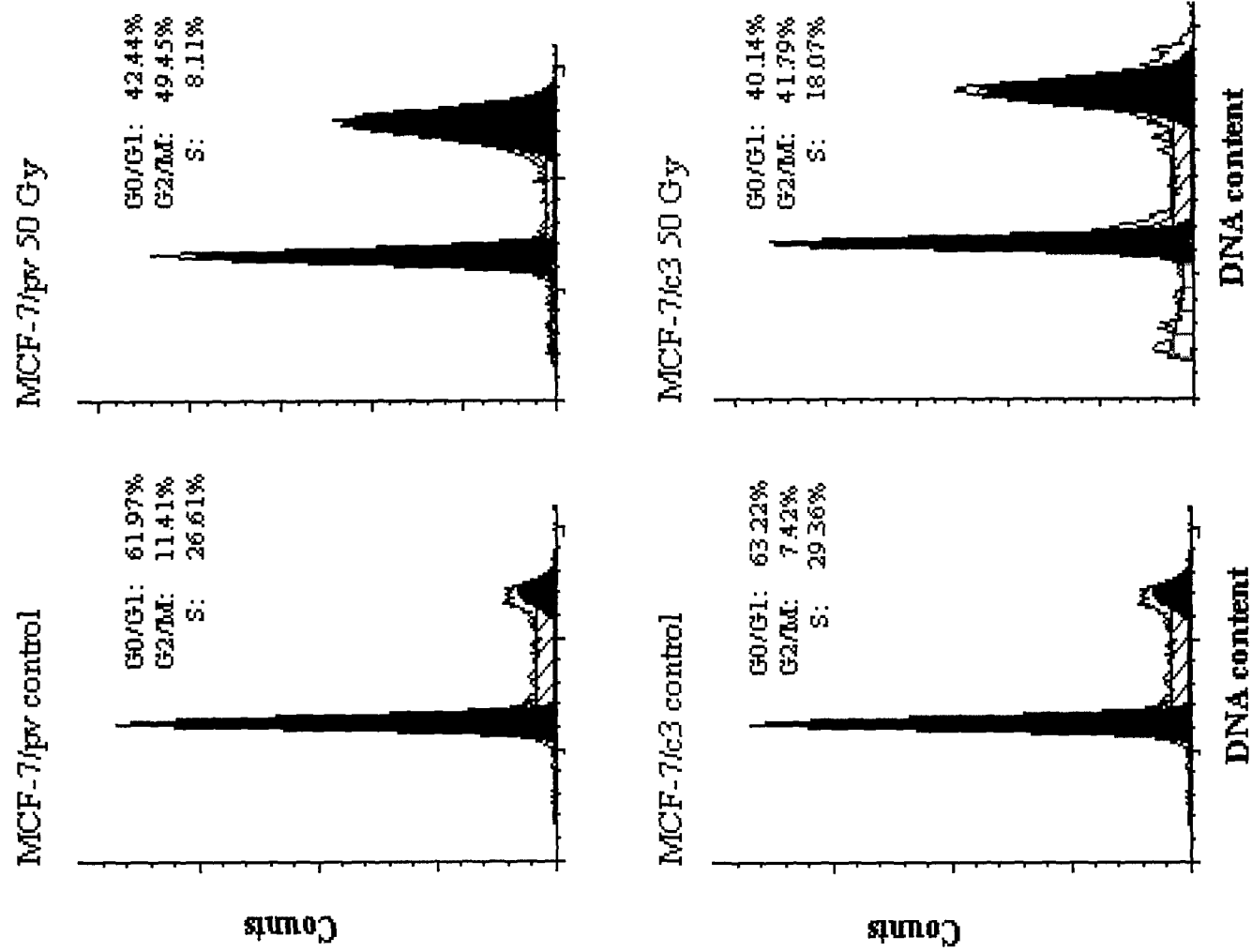
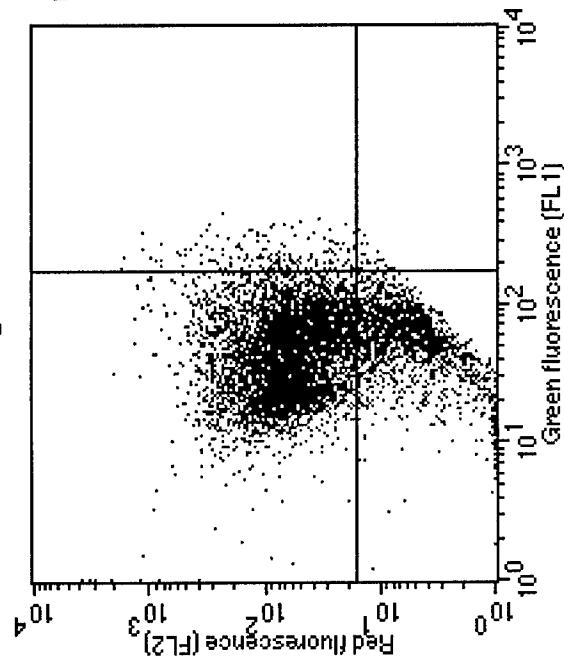
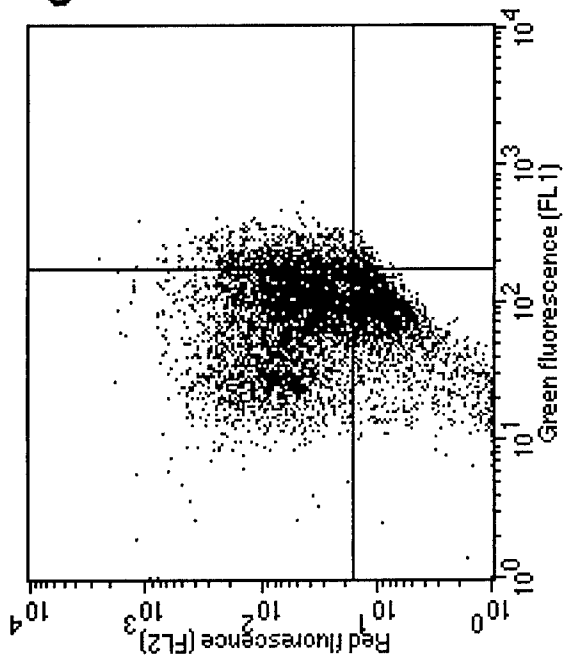


Figure 5

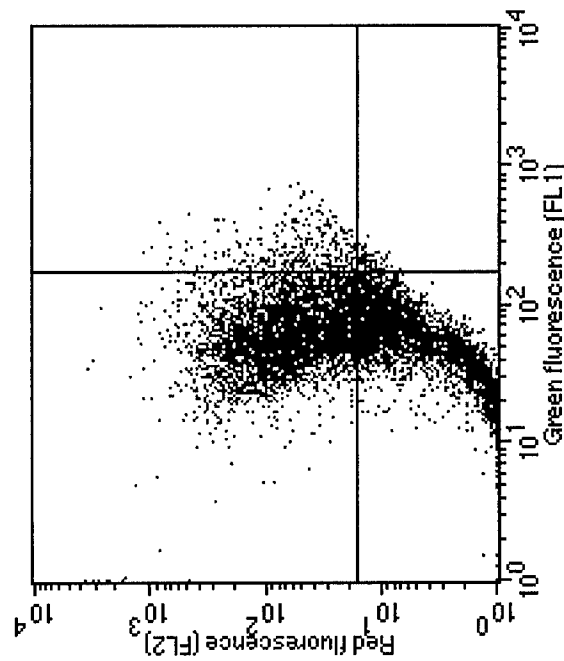
MCF-7/pv control



MCF-7/pv 50 Gy



MCF-7/c3 control



MCF-7/c3 50 Gy

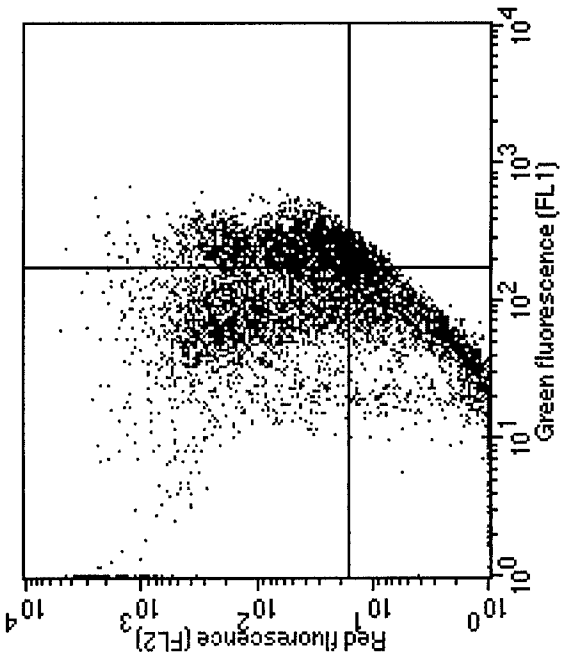


Figure 6

Appendix C

The role of p53 in caspase 3 activation during chemotherapy-induced apoptosis in the MCF-7 breast cancer cell line

Alison R. Johnson

Academic Research Thesis

The role of p53 in caspase 3 activation during chemotherapy-induced apoptosis in the MCF-7 breast cancer cell line

*Alison Johnson*¹

Abstract

Apoptosis, or programmed cell death, is a process to regulate the balance between cell proliferation and cell death, and consequently, it is an integral part of homeostasis. Apoptosis is mediated by various proteases, known as caspases, which cleave cellular substrates. Caspase 3 is a key caspase in the caspase cascade. Apoptosis is regulated by a number of factors including p53, a tumor suppressor gene. Although p53 is reported to be involved in apoptosis regulation, a detailed mechanism of p53-mediated activation of caspase 3 in chemotherapy-induced apoptosis is not clear. To study the role of p53 in caspase 3 activation during apoptosis, we have examined the correlation between p53 function and caspase 3 activation in MCF-7 cells. First, we examined the response of endogenous p53 and caspase 3 activation after treatment with chemotherapy agents. We found that both doxorubicin and etoposide are capable of inducing p53 increase and caspase 3 activation. However, it appeared that caspase 3 activation is not directly proportional to p53 levels, suggesting that while p53 is probably involved in caspase 3 mediated apoptosis, it is most likely not a factor determining the extent of apoptosis. Second, we studied the effect of disrupting endogenous p53 on caspase 3 activation. To do this, we established 4 new double transfectant stable cell lines with the dominant negative p53 mutant using the MCF-7/C₃ and MCF-7/PV cell lines. The preliminary studies on these new cell lines indicate that an interaction between p53 and caspase 3 does exist, but that future research is necessary to define the relationship more clearly.

Introduction

The homeostasis of a multicellular organism is dependent on the balance between cell proliferation and cell death (1). The process of programmed cell death, or apoptosis, is a mechanism of regulation that can eliminate cells that have been produced in excess, that have developed improperly, or that have genetic damage (2). Certain disorders are associated with increased cell survival, or an inhibition of apoptosis, while others are associated with increased apoptosis (1). Those that are characterized by decreased apoptosis include various types of cancers, autoimmune disorders, and certain viral infections. Conditions associated with increased apoptosis such as AIDS, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis could be attributed to an accumulation in signals that induce apoptosis, or a lower threshold at which these events occur. Clearly, apoptosis plays a crucial role in the pathogenesis of disease.

Apoptosis can be induced by various extracellular stimuli, including cytokines, DR ligands, such as TNF, fas, and TRAIL, as well as by intracellular stimuli such as chemotherapy and radiation (3). Apoptosis is characterized by cytoskeleton disruption, cell shrinkage, and membrane blebbing. In addition, endonucleases are activated to

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degrade nuclear DNA, and the condensed chromatin localizes near the edge of the nuclear membrane (4). Ultimately, the cell is broken up into small membrane bound fragments called apoptotic bodies, which are engulfed by neighboring cells. Apoptosis differs from another type of cell death called necrosis, where the cell membrane permeability is increased, leading to rapid swelling and lysis (4). While necrosis leads to an inflammation response, apoptosis does not.

Apoptosis is regulated by a number of proteins including, p53, a well-known tumor suppressor (5-8). p53 regulates the transcription of many genes. Some of these genes are involved in cell cycle arrest, and others are involved in apoptosis regulation. Consequently, appropriate activation of p53 would result in either cell cycle arrest and DNA repair or apoptosis. Both are significant mechanisms of maintaining homeostasis. The balance between these two functions takes many factors into consideration, including the extent of DNA damage. For instance, if the damage is repairable, the gene may induce cell cycle arrest, but if the damage is not repairable the cell will undergo apoptosis.

The p53 gene encodes a protein that acts as a transcription factor for p21, a cyclin-dependent kinase inhibitor which blocks cell cycle progression. Thus, p53 extends the time available for DNA repair before the cell enters into the S phase. Because p21 initiates G1 cell cycle arrest (9) as well as G2 cell cycle arrest (10), it interrupts the apoptosis pathway at a point downstream from p53 but upstream from caspase 3. Cells that do not have functional p21 may undergo apoptosis instead of cell cycle arrest. For instance, if p21 is cleaved by caspase 3 during p53-dependent apoptosis, cancer cells can be converted from undergoing cell cycle arrest to apoptosis, and consequently have increased sensitivity to chemotherapy drugs (11).

As mentioned above, another role of p53, besides inhibiting the cell cycle, is to initiate apoptosis induced by intracellular stimuli, such as chemotherapy-mediated DNA damage. It is believed that in p53 mediated apoptosis, p53 regulates apoptosis by inducing the transcription of other apoptotic regulators such as bax. By regulating these target genes, p53 could affect the permeability of the mitochondria, which results in the release of cytochrome c and caspase 9 activation. Caspase 9 then activates downstream caspases, such as caspase 3. While p53 is required for cells to initiate apoptosis in response to DNA damaging agents (12), it may not affect the overall extent of cell death induced by certain chemotherapy agents (13).

Various p53 mutations can have different effects on apoptosis and cell cycle arrest. Some p53 mutants have lost the ability to act as a transcription factor for certain target genes, however, they have retained their ability to undergo apoptosis (14). In this case, the cells undergo apoptosis rather than growth arrest (9). As a result of other p53 mutations, tumor cells are often unable to undergo apoptosis and have increased resistance to chemotherapy and radiation therapy. This leads to the possibility that p53 status might be an important determinant of response to therapy (15). Many types of cancers including lung, breast, colorectal, and prostate are often associated with p53 mutations and have poorer prognosis (16,17). Decreased apoptosis is a fairly new

mechanism of drug resistance (16); earlier theories included enhanced drug metabolism, drug target amplification, and repair of damaged targets.

Caspases are a group of proteases that mediate apoptotic execution (18, 19). They specifically cleave their substrate after an aspartic acid residue. The apical caspases (2, 8, 9, and 10) are responsible for the initiation of apoptosis, while the effector, or downstream, caspases (3, 6, and 7) actually execute apoptosis because they cleave cellular substrates, including poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme, DNA fragmentation factor, lamin β 1, topoisomerase 1, β actin and actin. Caspases are expressed in an inactive form (procaspase) and are not activated until the prodomain is cleaved and the large and small fragments associate with each other as a heterodimer. The apical caspases typically lead to the activation of effector caspases. The sequence of events leading to programmed cell death is known as the caspase cascade.

Caspase 3, also known as CPP32, YAMA, or apopain, is a key caspase in this cascade leading to apoptosis (20). Caspase 3, like all of the caspases, is related to the interleukin 1 β -converting enzyme (ICE) and CED-3, which is required for apoptosis in *Caenorhabditis elegans* (21). Caspase 3 is expressed in the inactive form and is activated after it is cleaved into a large (p20) and small (p10) fragment, which then associate as a heterodimer (21). Caspase 3 preferentially cleaves cellular substrates at the sequence Asp-Glu-Val-Asp (DEVD). Caspase 3 is mainly responsible for the cleavage of PARP (22), but also cleaves the 70-kD protein component of the U1-ribonucleoprotein and the catalytic subunit of the DNA dependent protein kinase (23).

p53 and caspase 3 interact during the apoptosis cascade induced by DNA damaging agents. Yu and Little (1998) investigated ionizing radiation induced apoptosis in three different lymphoblast cell lines that differ in p53 status. While cells with wtp53 underwent rapid apoptosis, the response was reduced and delayed in cells without functional p53. These differences in apoptosis correlated with early phase caspase 3 activation. These results suggest that early activation of caspase 3 depends on p53, however, the later activation of caspase 3 may not involve p53. p53 may be involved for caspase 3 activation, but is not required (24). Fuchs et al. (1997) investigated whether p53 dependant apoptosis requires activation of caspase 3 in thymocytes, and found that the p53 mediated activation of caspase 3 might be independent of the fas/fasL interaction (25). Furthermore, caspase 3 activation is a key component of p53-induced apoptosis in neurons, and if apoptosis is initiated in bax-deficient cells, the neurons did not exhibit caspase 3 activation in response to p53, and were protected from apoptosis. It appears that, in neurons, caspase 3 activation is dependent on bax (26).

Although p53 is reported to be involved in apoptosis regulation, since apoptosis is also regulated by other factors, how much p53 contributes to caspase 3 activation in chemotherapy induced apoptosis is not clear. Past studies in this lab have focused on the specific role of caspase 3 in chemotherapy induced apoptosis in the MCF-7 breast cancer cell line, which is deficient in caspase 3 and expresses the wtp53 (27). To do this, two cell lines have been established. One is the MCF-7 cell line reconstituted with caspase 3

(MCF-7/C₃); the other is the MCF-7 cell line that has been transfected with an empty vector (MCF-7/PV), which is caspase 3 deficient. Using these cell lines, studies have shown that caspase 3 reconstitution sensitizes MCF-7 cells to chemotherapy and radiation induced apoptosis (28)

This study examines the role of p53 in caspase 3 activation during chemotherapy-induced apoptosis in the MCF-7 breast cancer cell line. Although many factors are most likely involved in caspase 3 activation such as Bcl-2, Bax, ceramide, and caspase 8, we examined the role of p53 in this sensitization to chemotherapy. We know that caspase 3, a downstream effector in caspase execution, is affected by the concentration of chemotherapy, but we want to discover the role of p53 in this mechanism. First, we examined the response of endogenous p53 and caspase 3 activation after etoposide or doxorubicin treatment in MCF-7/PV and MCF-7/C₃ cells. We found that while both drugs caused an increase in p53 levels, the caspase 3 activation was not directly proportional to these increases. To further explore the interaction between p53 and caspase 3 we established 4 new double transfectant cell lines (MCF-7/PV/pcmV; MCF-7/PV/mtp53; MCF-7/C₃/pcmV; MCF-7/C₃/mtp53). The MCF-7/PV and MCF-7/C₃ cells were transfected with either the dominant negative mtp53 gene or the control plasmid. These results suggest that an interaction between p53 and caspase 3 does exist, however, further research, such as a transient transfection of the mtp53 gene, should be done to understand the relationship more clearly.

Methods

Cell Culture

MCF-7 cells were maintained in IMDM medium supplemented with 10% fetal bovine serum (FBS) and penicillin/ streptomycin, and stored in a 5% CO₂ incubator.

PCMV Plasmid Construction

To prepare the pcmV plasmid the pcmV mtp53 plasmid (Clontech, Palo Alto, CA) was transformed into *e.coli* cells and the resulting clones were grown on neomycin plates and then purified using the Qiagen QIAprep Spin Miniprep Kit (Qiagen Inc, Valencia, CA). After confirming the presence of the plasmid in the clone using agarose gel electrophoresis, the plasmid was digested with EcoRI and HIND III to remove the mtp53 insert. Another gel was run to confirm the appropriate fragmentation pattern, and then the large fragment was isolated. Blunt ends on the plasmid were created using T₄ polymerase, and the plasmid was ligated with T₄ ligase.

Transfection

The control pcmV plasmid or the pcmV vector containing the mtp53 gene (PCMVmtp53) were transfected into MCF-7/PV and MCF-7/C₃ cells. The protocol from Life Technologies was followed for stable transfection of adherent cells. Cells were incubated in a CO₂ incubator at 37°C until the cells were 80% confluent. 1 µg of DNA was diluted in 100 µl OPTI-MEM® I Reduced Serum Medium (GIBCO BRL™) and combined with 25 µl LIPOFECTAMINE Reagent (Life Technologies, Rockville, MD) diluted in 100 µl

of serum free medium. The solution was mixed gently and then incubated at 45 minutes, to allow DNA-liposome complexes to form. .8 ml of serum free medium was added to the tubes containing the liposomes and this was applied to the monolayer of cells. Fresh medium was added to the cells 24 hours after transfection. Stable transformants were selected using G418, and confirmed using a western blot.

Western Blot

Cells were treated with the properly diluted drug (etoposide or doxorubicin (DOX)) 18 hours before collection. After being washed with PBS, the treated cells were resuspended in lysis buffer, and incubated on ice for 45 minutes. Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). 30µg of lysate was separated in a 12% SDS-page gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk in TBS-T washing buffer and probed with the specific antibody. After the final washing with TBS-T, ECL reagent (Amersham-Pharmacia Biotech, Inc, Piscataway, NJ) was added to the membrane. The membrane was visualized by autoradiography.

DEVD cleavage assay

Cells were treated with the properly diluted drug (etoposide or DOX) 18 hours before collection. The collected cells were washed with PBS and the cells were resuspended in 360µl of DEVD lysis buffer (50mM pH 8.0 Tris-HCl, 130 mM KCL, 1mM EDTA, 10 mM EDTA, 10mM EGTA, and 10µM digitonin) and incubated for 10 minutes at 37°C. Samples were spun at 5000 rpm for 5 minutes and the supernatant was collected. 100µl of the clarified lysate was added in triplet to a fluorometer plate, and then 100µl of the substrate DEVD-AMC (BD PharMingen, San Diego, CA) was added to each well. The samples were read every 10 minutes, up to one hour, using a fluorometer. The remaining lysate was used to calculate protein concentration using BCA, and the results were adjusted for the detected differences.

Results

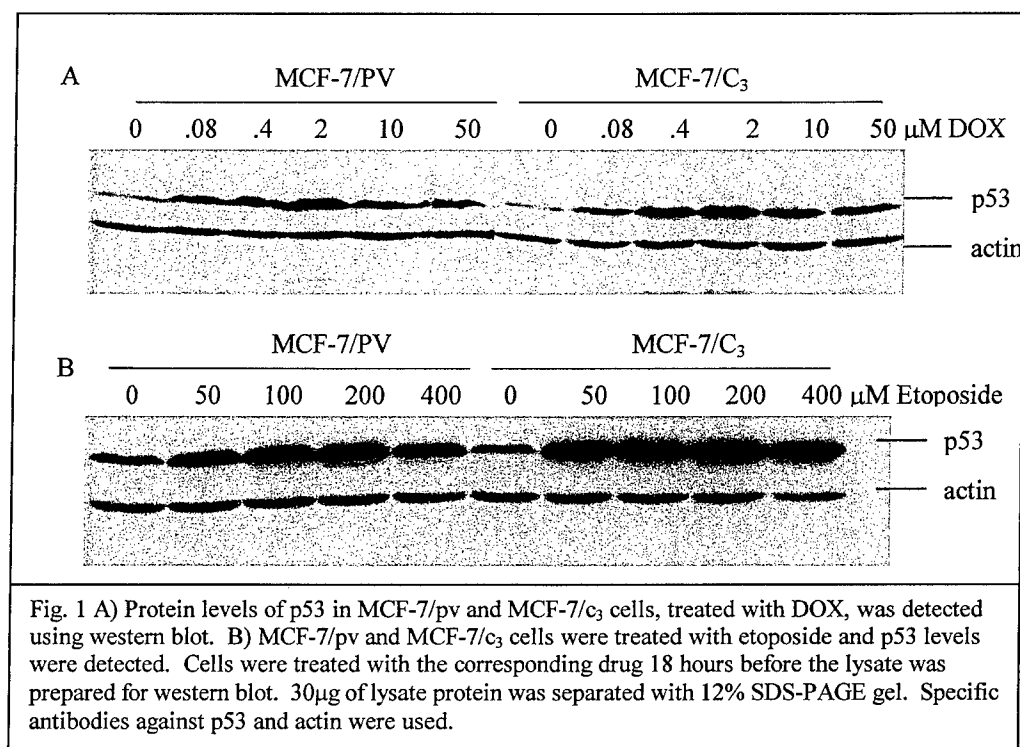
A. The response of endogenous p53 levels and caspase 3 activation in chemotherapy treated MCF-7/PV and MCF-7/C₃ cells

MCF-7/PV and MCF-7/C cells were treated with various chemotherapy drugs to determine whether the increases in p53 levels were correlated with increases in caspase 3 activation. These experiments can help us to elucidate the mechanisms by which DOX and etoposide induce cell death. Chemotherapy drugs typically activate p53, which then initiates apoptosis. However, it is still not clear how much p53 activates caspase 3 between the various drugs.

1. Increase in endogenous p53 levels in cells treated with DOX or etoposide

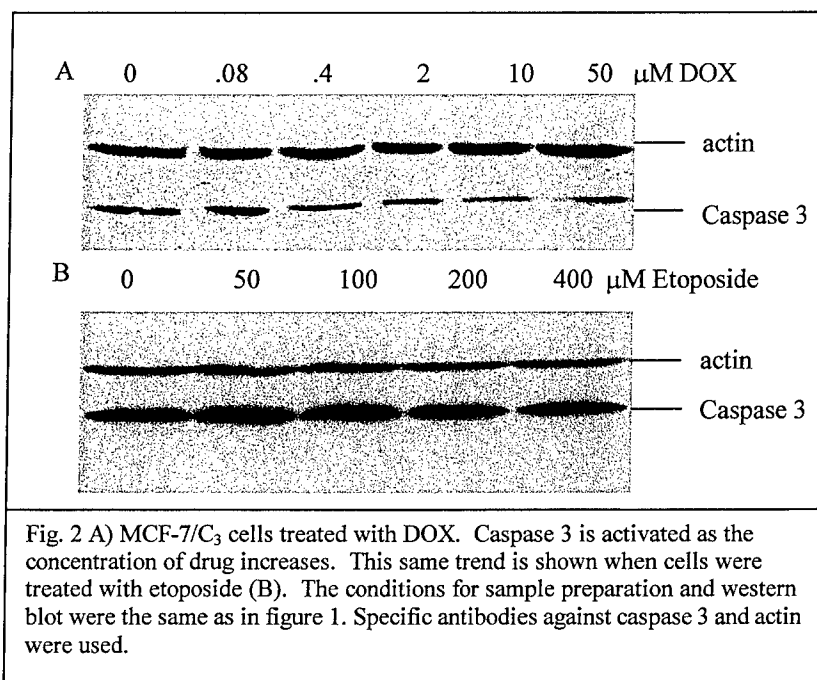
MCF-7/PV and MCF-7/C₃ cells were treated with various concentrations of DOX (0, 0.08, 0.4, 2, 10, and 50 µM), and the levels of p53 expression were detected using a western blot (Fig. 1A). The level of p53 protein expression increased with drug

treatment. At higher level of drug (10 and 50 μ M DOX), it appears that p53 levels go down. However, this can be attributed to an increase in cell death at these levels, and consequently, a lower number of cells in which to detect the p53 levels. p53 levels were also detected by western blot after MCF-7/PV and MCF-7/C₃ cells were treated with various concentrations of etoposide (0, 50, 100, 200 and 400 μ M) (Fig. 1B). The similar trend of increased p53 levels in cells treated with drug as compared to controls was observed.



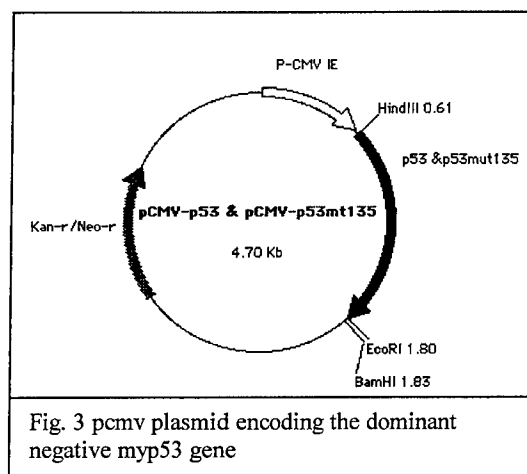
2. Caspase 3 activation in DOX or Etoposide treated MCF-7/C₃ cells

Caspase 3 activation was examined by western blot in MCF-7/PV and MCF-7/C₃ cells treated with either DOX (Fig. 2A) or etoposide (Fig. 2B). Both drugs were capable of initiating caspase 3 mediated apoptosis, as shown by increased activation of caspase 3 under increasing concentrations of either drug. The antibody used in these experiments recognizes the inactive, or procaspase, form of caspase 3, so a weaker signal indicates more cleavage, or activation, of caspase 3.



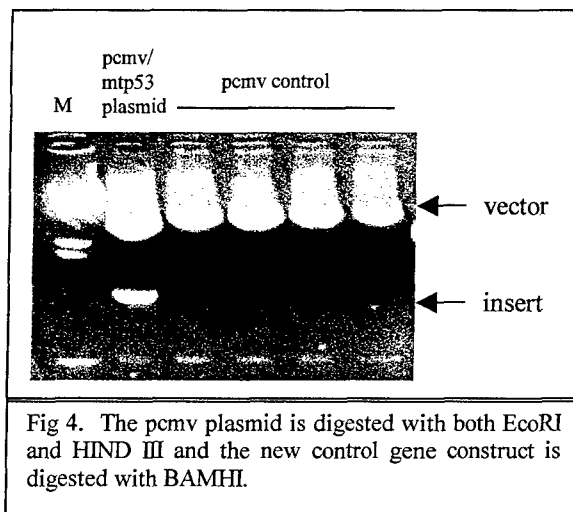
B. Effects of disruption of endogenous wtp53 and caspase 3 activation in MCF-7/PV cells and MCF-7/C₃ cells.

To further explore this interaction between p53 and caspase 3 we established 4 new double transfectant stable cell lines (MCF-7/PV/pcm, MCF-7/PV/mtp53, MCF-7/C₃/pcm, and MCF-7/C₃/mtp53). Endogenous p53 would be disrupted by transfecting the MCF-7/PV and MCF-7/C₃ cells with either the dominant negative mutant p53mt135 encoded in the pcmv plasmid (Fig. 3) or a control pcmv plasmid. This mtp53 differs from the wtp53 because of a G to A conversion at nucleotide 1017, which causes a conformational change. When wtp53 and mtp53 are co expressed, they form a mixed tetramer that cannot interact with the appropriate binding sites. Because this binding is inhibited, downstream effects of p53 are blocked. We were interested in the decrease of caspase 3 activation that occurs after disruption of p53 function.



1. PCMV control plasmid construction

The control plasmid was confirmed after digestion of both the original pcmv plasmid with EcoRI and Hind III and the new construct with BamHI (Fig 4). The large fragment of the intact plasmid should be nearly the same size as the control plasmid digested with BamHI. This result serves as a confirmation that the construct was in fact ligated correctly because the control vector is identical to the original plasmid, except for the excision of the mtp53 gene.



2. Establishment of stable MCF-7/PV and MCF-7/C3 cell lines overexpressing dominant negative mtp53

Four new, double transfectant cell lines were established, by transfecting the MCF-7/PV and MCF-7/C₃ cells with either the pcmv control or dominant negative mtp53 plasmid. The cell lines and their characteristic properties are summarized in table 1 below.

Table 1: Properties of the cell lines

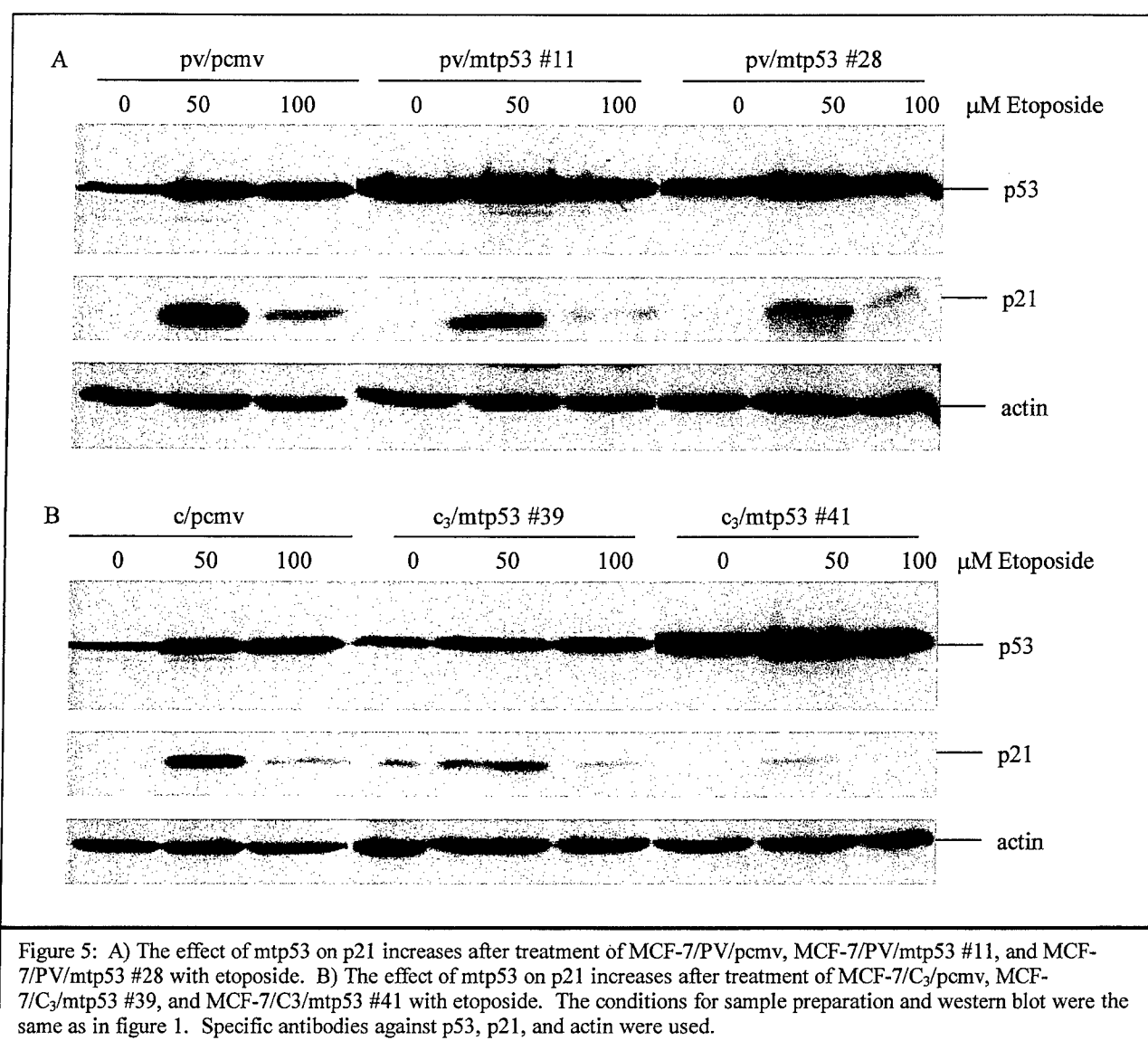
Cell Line	Caspase 3	mtp53
MCF-7/pv/pcmvm	-	-
MCF-7/pv/mtp53	-	+
MCF-7/c ₃ /pcmvm	+	-
MCF-7/c ₃ /mtp53	+	+

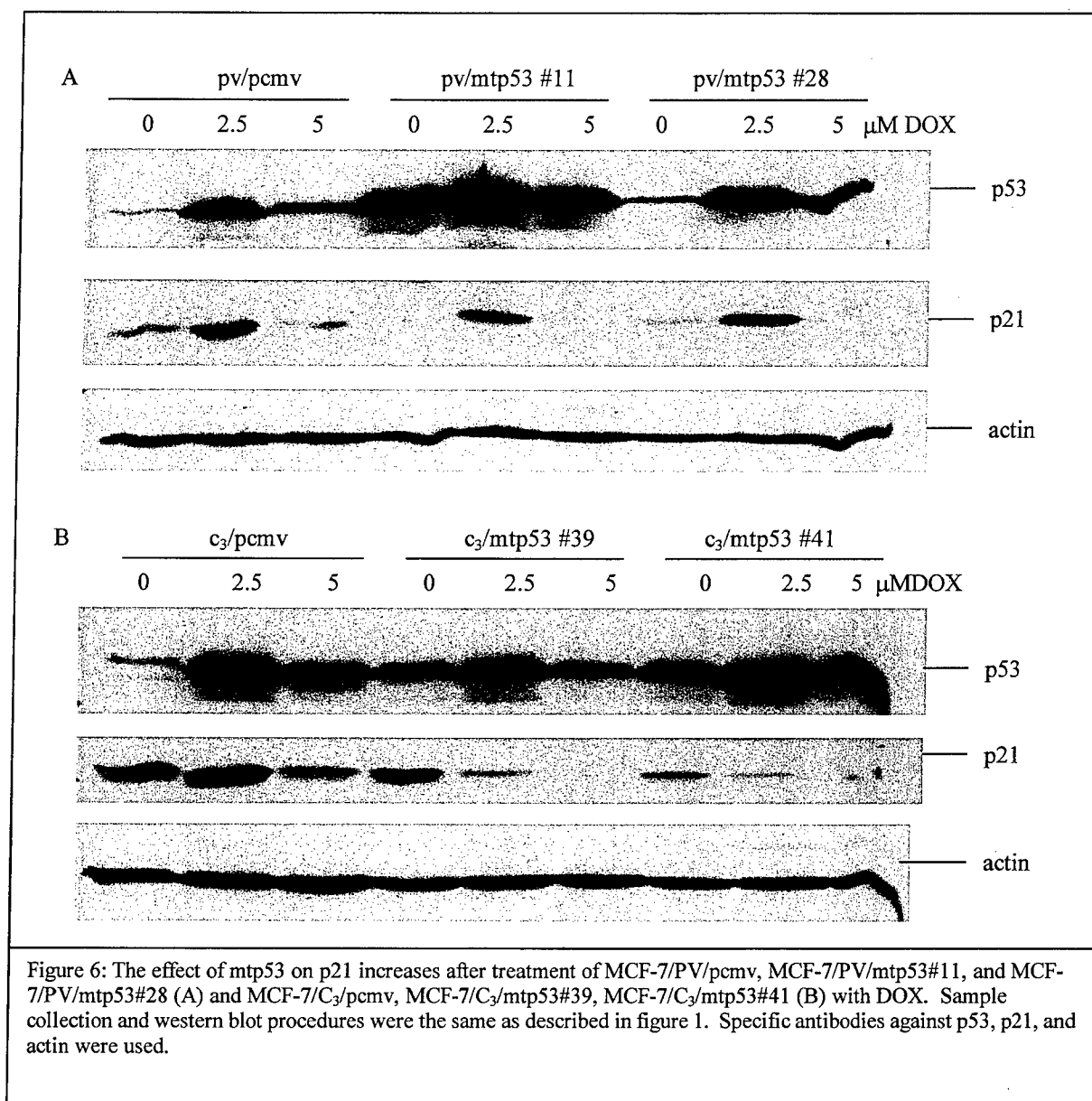
MCF-7/PV/pcmvm, MCF-7/PV/mtp53 #11, MCF-7/PV/mtp53 #28, MCF-7/C₃/pcmvm, MCF-7/C₃/mtp53 #39, and MCF-7/C₃/mtp53 #41 were selected for further analysis, because of their high levels of p53 as detected in a western blot.

3. p53 levels and disruption of p53 function in MCF-7 cells expressing dominant negative mtp53

All cell lines mentioned above were treated with etoposide and there was an increase in p53 levels with drug treatment, as detected by western blot (Fig. 5). These cell lines were also treated with DOX (0, 2.5, or 5 μ M), and a similar increase in p53 levels was observed (Fig. 6). The antibody used for p53 detects both wt53 and mtp53. We know from the western blot that the levels of p53 are higher after transfection with the dominant negative mtp53 than with transfection of the control pcmv plasmid. However, we needed to establish that the introduction of this new gene actually disrupted the

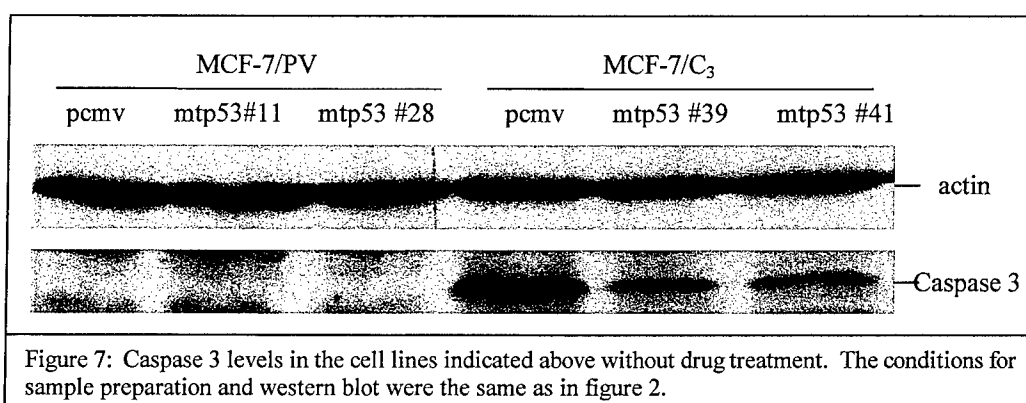
function of the p53 protein. One of the targets of p53 is p21, so that in cells with wtp53, an increase in p53 levels should be correlated to an increase in p21 levels. Therefore, if p53 function is disrupted there should be an increase in p21 levels after drug treatment in the control cell lines, but not in the cell lines with the mtp53 gene. Using a western blot, we detected the p53 and p21 levels following either treatment with etoposide or DOX. In both cases, while the increase in p21 was quite large from either 0 μ M to 50 μ M etoposide or from 0 μ M to 2.5 μ M DOX in the control cell lines, the increase was not as large in cell lines with the mtp53. These findings are particularly significant in the clones MCF-7/PV/mtp53 #11, and MCF-7/C₃/mtp53 #41, reflecting the increased expression of the dominant negative mtp53 and disruption of p53 function. These results suggest that p53 function is disrupted in the cell lines transfected with mtp53.





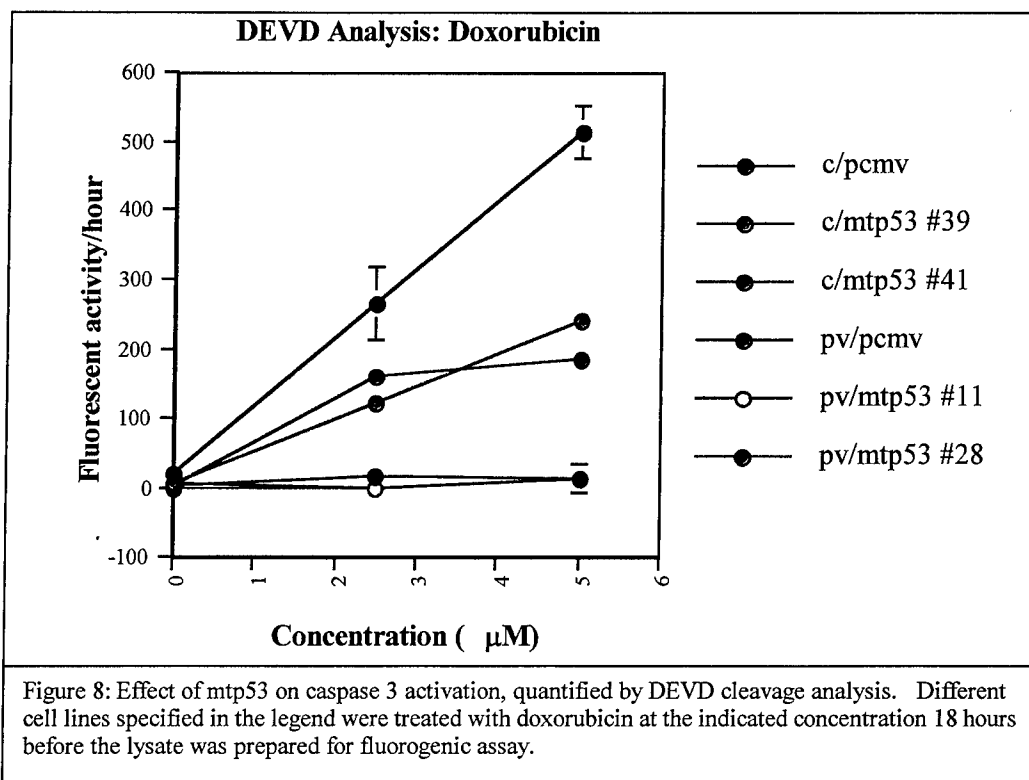
Caspase 3 levels in untreated cells

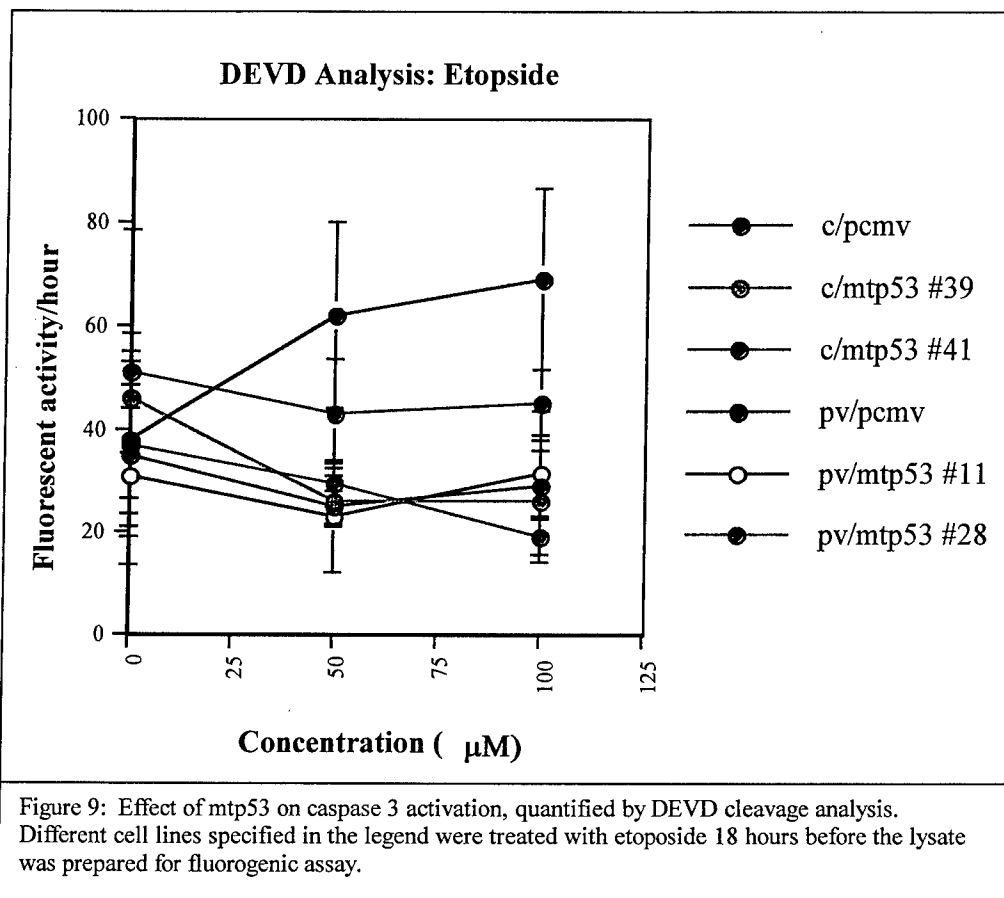
Before further characterization of dominant negative mtp53 cell lines, we detected the caspase 3 levels in MCF-7/C₃ cells (Fig. 7). To our surprise, C₃ levels in mtp53 overexpressing MCF-7/C₃ cells were lower than that in MCF-7/C₃/pcmv cells. Since all cell lines were derived from the same MCF-7/C₃ clone, the result suggests that in the process of selecting the mtp53 cell lines, high level of caspase 3 and high level of mtp53 were not compatible.



Caspase 3 activation

To detect the response of caspase 3 and mtp53 overexpressing cells to chemotherapy treatment, caspase 3 activation was quantified using DEVD cleavage analysis. This is a more sensitive and quantitative way to examine caspase 3 activation than a western blot. Because caspase 3 cleaves cellular substrates with the DEVD sequence, this assay allows us to examine caspase 3 activity in cells treated with drugs. We expected that the levels of caspase 3 activation, and consequently fluorescence, should be lower in the cell lines with the mtp53 gene, than the control cell lines. The DEVD cleavage activity after DOX treatment is shown in figure 8 and DEVD cleavage after etoposide treatment is shown in figure 9. In both experiments, the DEVD cleavage of MCF-7/PV derived cell lines was quite low, which is consistent with their deficiency in caspase 3. With respect to the cell lines that contained caspase 3, there was increased activation of caspase 3 in the control cell lines as compared to the cell lines expressing mtp53. These results suggest that disruption of p53 function significantly decreases the amount of caspase 3 mediated apoptosis.





Discussion

Research has indicated that p53 is required for cells to initiate apoptosis in response to DNA damaging agents. These results are consistent with the concept that chemotherapy drugs such as etoposide and DOX induce DNA damage, which then activates p53. While the levels of p53 increase are quite similar in DOX and etoposide treated cells, it appears that DOX may utilize more of a caspase 3 mediated pathway during cell death, because there is greater activation of caspase 3 in DOX treated cells. These findings were further supported by evidence from the DEVD cleavage assays. In the cells treated with DOX, there is a substantial difference in the amount of DEVD cleavage between the control cells and those with the mtp53 gene. While this trend is observed in etoposide treated cells, it is not as drastic. This again suggests that doxorubicin may initiate apoptosis through more of a caspase 3 mediated mechanism than etoposide.

Previous studies in the MCF-7 breast cancer cell line have suggested that disruption of endogenous p53 may sensitize cells to some DNA damaging agents (29). This increased cell death was attributed to defects in the G1 checkpoint. The results of the DEVD analysis indicated that there is less caspase 3 activation in cells with the mtp53 gene,

which suggests that activation of caspase 3 is critical for the p53 dependant apoptosis. However, it is essential to consider that this result reflects the combination of differing levels of caspase 3 in the cells without drug treatment as well as the impact of the mtp53 gene. These results suggest that there truly is an interaction between p53 and caspase 3, because during the selection process mtp53 and caspase 3 are not compatible. We detected that after the selection process, caspase 3 levels were lower in the cells transfected with mtp53. A possible explanation for this finding is that cells that had disrupted endogenous p53 function as well as high levels of caspase 3 may have been stimulated to undergo spontaneous cell death.

Clearly, there is some interaction between p53 and caspase 3 and these preliminary findings will stimulate future work in the lab. The next step would be to conduct a transient transfection of mtp53, so that the cells will not have to undergo a lengthy selection process, where caspase 3 levels will decrease. Then, we can better determine the role of p53 in caspase 3 mediated apoptosis. Characterization of the apoptosis pathway and knowledge of the interactions between caspase 3 and p53 may have implications for optimal cancer chemotherapy. For instance, we see that DOX and etoposide utilize different pathways to cause apoptosis and certain patients may benefit more from a particular drug, depending on whether the tumor has caspase 3 or is caspase 3 deficient. We know that caspase 3 plays a major role in apoptosis, and perhaps gene therapy with the caspase 3 gene could become a reality. This could maximize the therapeutic efficacy in patients with defined genetic alterations.

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Appendix D

Reconstitution of Caspase 3 Sensitizes MCF-7 Breast Cancer Cells to Doxorubicin- and Etoposide-induced Apoptosis¹

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ABSTRACT

MCF-7, a breast cancer-derived cell line, is deficient of caspase 3 and relatively insensitive to many chemotherapeutic agents. To study the association of caspase 3 deficiency and chemotherapeutic resistance, we reconstituted caspase 3 in MCF-7 cells and characterized their apoptotic response to doxorubicin and etoposide. Western blots demonstrated that caspase 3 was constitutively expressed in the reconstituted MCF-7 cells. Both morphological observation and survival assays showed that caspase 3 reconstitution significantly sensitized MCF-7 cells to both drugs. Remarkably increased activation of caspases 3, 6, and 7, cleavage of cellular death substrates, and DNA fragmentation were detected in the reconstituted MCF-7 cells after drug treatment. Together, these data demonstrated a specific role for caspase 3 in chemotherapy-induced apoptosis and in activation of caspases 6 and 7. Our results also suggest that caspase 3 deficiency may contribute to chemotherapeutic resistance in breast cancer.

INTRODUCTION

Chemotherapeutic resistance is a major problem in human oncology. Mechanisms of chemotherapeutic resistance are diverse and poorly defined for most cancer subtypes. Recent studies suggest that aberrant apoptosis (programmed cell death) likely contributes to this process (1). Apoptosis is a genetically controlled process that can be triggered by different extracellular and intracellular stimuli (2). Apoptotic execution requires coordinated activation of a special group of proteases, known as caspases (3, 4). The activation of caspases is a signaling cascade mediated by proteolysis (5). Activated caspases subsequently cleave cellular death substrates and cause biochemical and morphological changes, leading to apoptosis (6). Fourteen mammalian caspases have been cloned (4, 7). Caspases 2, 8, 9, and 10 (apical caspases) initiate apoptosis and activate downstream caspases. Caspases 3, 6, and 7 (effector caspases) are activated by apical caspases and further cleave cellular death substrates (4).

Caspase 3 (also known as cpp32, yama, and apopain) is a key caspase in this signaling cascade (8-12). Caspase 3 activity has been detected in apoptosis induced by a variety of apoptotic signals, including death receptor activation (13), growth factor deprivation (14), ionizing radiation (15), and treatment with granzyme B (16) or different chemotherapeutic agents (17). Caspase 3 knockout mice displayed abnormal brain tissue development due to lack of apoptosis (18). A growing number of substrates cleaved by caspase 3 have been

identified, such as PARP³ (10), sterol-regulatory element-binding protein (19), gelsolin (20), the U1-associated *M_r* 70,000 protein (21), D4-GDI (22), DFF (23), DNA-dependent protein kinase δ and θ (24, 25), α -fordrin (26), and huntingtin (27). Caspase 3 is believed to play a pivotal role in apoptotic execution.

Alterations in apoptosis-associated genes are often observed in cancers. The *p53* tumor suppressor gene, a key regulator in DNA damage-induced apoptosis, is frequently mutated in human tumors (28). Overexpression of apoptotic inhibitors, such as bcl-2 and bcl-x_L (29, 30), and down-regulated apoptosis-promoting factors, such as Bax- α and Fas (31, 32), has been detected in primary tumors and tumor cell lines. These alterations have been linked to chemotherapeutic resistance (31, 33). Correction of these alterations has resulted in sensitization of the defective cells to chemotherapeutic agents (34).

Caspase 3 deficiency was recently detected in MCF-7 breast cancer cells. It is due to a deletion mutation in exon 3 of the gene (35). Overexpression of caspase 3 in MCF-7 cells indicates that caspase 3 plays a critical role in both death receptor- and mitochondria-mediated apoptotic pathways (35-38). Given the important role of caspase 3 in apoptotic execution and the correlation between the alterations of other apoptotic regulators and chemotherapeutic resistance, we postulated that caspase 3 deficiency might also significantly contribute to chemotherapeutic resistance. Although caspase 3-like activity has been detected in the apoptosis induced by various chemotherapeutic drugs (17), the specific role of caspase 3 in this process warrants further investigation due to the overlapping activities among effector caspases (18, 39). To evaluate the role of caspase 3 in chemotherapy-induced apoptosis, we reconstituted caspase 3 in MCF-7 cells and characterized the apoptotic responses of the MCF-7 cells to doxorubicin and etoposide in comparison with control cells. We found that reconstitution of caspase 3 significantly sensitized MCF-7 cells to doxorubicin- and etoposide-induced apoptosis.

MATERIALS AND METHODS

Cell Culture, Plasmid Construction, and Transfection. MCF-7 cells were maintained in Iscove's modified Dulbecco's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The pBabepuro/caspase 3 plasmid was constructed by treating the *Bam*HI/*Pst*I caspase 3 cDNA insert from pBS/caspase 3 plasmid (a gift from Drs. David Boothman and John Pink) with T4 DNA polymerase and then subcloning it into the blunt-ended pBabe/puromycin retroviral vector (40). MCF-7 cells were placed into 60-mm dishes at 3×10^5 cells/dish and allowed to grow overnight. Two μ g of caspase 3 encoding pBabepuro plasmid were mixed with 10 μ l of LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) and transfected into the cells according to the manufacturer's instructions. Empty pBabepuro vector was also transfected as a control. Twenty-four h after transfection, the cells were trypsinized, diluted, and placed into 96-well plates. Transfected cells were then selected with 2 μ g/ml puromycin. Individual puromycin-resistant clones were screened for caspase 3 by Western blot. Five caspase 3-positive clones were pooled for further characterization. Morpho-

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³The abbreviations used are: PARP, poly(ADP-ribose) polymerase; DFF, DNA fragmentation factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4',6-diamidino-2-phenylindole.

logical changes were observed and photographed with a phase-contrast microscope.

Drug Treatment and Sample Collection. For doxorubicin (Bedford Laboratories, Bedford, OH) and etoposide (Bristol-Myers Squibb Co., Princeton, NJ) treatments in studies other than the MTT assay (see below), 1×10^6 cells were seeded into 60-mm dishes 24 h before drug treatment. Various doses were added to the dishes 18 h before cell collection. Treated cells to be analyzed by flow cytometry and DAPI staining were trypsinized. Cells to be analyzed by DEVD (Asp-Glu-Val-Asp) cleavage assay and Western blot were scrapped off the dish. In all cases, medium from individual dishes, which might contain floating dead cells, was collected and mixed with the cell pellet from the same dish.

MTT Survival Assay. Three hundred cells were placed into each well of 96-well plates. Twenty-four h later, the medium was replaced with new medium containing defined doses of doxorubicin or etoposide. Six days after treatment, the medium was changed with phenol red-free medium containing 500 $\mu\text{g/ml}$ MTT (Sigma). Three h after incubation, MTT-containing medium was removed. The incorporated dye was dissolved in 100 $\mu\text{l/well}$ DMSO, and the plates were read at the wavelength of 570 nm using an ELISA reader. Absorbance in the treated cells was expressed as a percentage of control. Eight parallel samples were treated in each concentration point. Five separate experiments were performed.

DEVD Cleavage Assay. Drug-treated cells were washed with PBS and resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 130 mM KCl, 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin] at 320 $\mu\text{l/60-mm}$ dish. After incubation at 37°C for 10 min, the samples were spun for 3 min (5000 rpm), and the supernatant was collected. After adding 100 μl of lysate to each well of a fluorometer plate, 100 μl of substrate solution, 2 μM DEVD-AMC (PharMingen, San Diego, CA) in lysis buffer was added right before the reading. Fluorescence was measured in a microplate fluorometer (Cambridge Technology, Cambridge, MA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Results are reported as the fluorogenic activity over 1 h (T_{60} to T_0). Samples were prepared in triplicate.

Western Blot. PBS-washed cells were treated with lysis buffer (41) on ice for 30 min. Lysed cells were centrifuged at 14,000 rpm for 10 min to remove cellular debris. Protein concentrations of the supernatant were determined using BCA Protein Assay (Pierce, Rockford, IL). Fifty μg of cell lysate were loaded onto each lane of a gel. Protein was separated by either 10% or 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBS-T (5% milk in Tris-buffered saline-Tween 20) washing buffer (41) and probed with specific primary antibodies. Concentrations of the primary antibodies used ranged from a 1:500 dilution to a 1:2,000 dilution. Antibodies against caspases 3 and 7 were purchased from Transduction Laboratories (Lexington, KY). Antibodies against caspase 6 and DFF were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-lamin B antibody was from PharMingen. The anti-PARP antibody was from Boehringer Mannheim (Indianapolis, IN). Washed membranes were then probed with horseradish peroxidase-labeled antimouse, antirabbit, or antigoat secondary antibodies (Amersham Pharmacia, Arlington Heights, IL), respectively. The membranes were washed again and treated with enhanced chemiluminescence reagents (Amersham Pharmacia). The specific protein bands were visualized by autoradiography (41).

Flow Cytometry. Drug-treated cells were trypsinized and washed with PBS. The cells were then fixed in 50 μl of 0.125% paraformaldehyde in PBS at 37°C for 5 min, followed by the addition of 450 μl of ice-cold methanol to each sample. After being washed three times with PBS containing 0.1% Triton X-100 and treated with RNase A (0.04 Kunitz units) for 30 min, the cells were stained with 50 $\mu\text{g/ml}$ propidium iodide. Cell analysis was performed using a Coulter Epics 751 flow cytometer. The fraction of the total cell population present in the G_1 , S phase, G_2 -M phase, and hypodiploid peak was obtained from DNA histograms by mathematical modeling using MPLUS software (41).

Nuclear Staining. Drug-treated cells were collected and washed with PBS followed by fixation with 2% paraformaldehyde at 4°C for 30 min. The cells were stained with 0.5 $\mu\text{g/ml}$ DAPI for 30 min. Stained cells were then washed and mounted on slides using a cytospinner. Nuclear morphology of the cells was visualized using an Olympus fluorescence microscope.

IC₅₀ Determination and Statistical Analysis. For IC₅₀ determination from the MTT assay, nonlinear regression analysis was performed with Cricket

Graph software to generate curves for IC₅₀ calculation. Significance evaluation was performed by the paired *t* test.

RESULTS

Stable Reconstitution of Caspase 3 in MCF-7 Breast Cancer Cells. To reconstitute caspase 3, MCF-7 cells were transfected with pBabe/puro retroviral vector plasmid encoding a full-length procaspase 3 cDNA or empty vector as a control. After puromycin selection, MCF-7 cell lines reconstituted with caspase 3 (MCF-7/c3) and control cells transfected with pBabe/puro vector (MCF-7/pv) were obtained. As shown in Fig. 1, the protein levels of caspase 3 in MCF-7/pv, MCF-7/c3, and Jurkat cell controls were detected by Western blot. The caspase 3-specific antibody detected a strong protein band with a molecular weight of about 32,000 in MCF-7/c3 and Jurkat cells but not in MCF-7/pv cells. Caspase 3 levels reconstituted in MCF-7/c3 cells were comparable to those in Jurkat cells that express high levels of caspase 3. The results below demonstrate that the reconstituted caspase 3 was functional. We have since maintained MCF-7/c3 cells in culture for over 1 year, and the cells have shown stable expression of high levels of caspase 3.

Reconstitution of Caspase 3 Sensitizes MCF-7 Cells to Doxorubicin- and Etoposide-mediated Killing. To compare the sensitivity of MCF-7/c3 cells and MCF-7/pv cells to doxorubicin and etoposide, we studied the viability and morphological changes of treated cells. The IC₅₀s were determined using MTT assays, in which MCF-7/pv and MCF-7/c3 cells were exposed to 0.5–37 nM doxorubicin or 0.05–1 μM etoposide for 6 days. The sensitivities of MCF-7/pv and MCF-7/c3 cells to each drug are shown in Table 1. The results indicated that MCF-7/c3 cells were significantly sensitized to both drugs ($P < 0.01$ for doxorubicin; $P < 0.05$ for etoposide). This suggests that caspase 3 reconstitution sensitized MCF-7 cells to doxorubicin and etoposide treatments.

Morphological changes commensurate with striking cytopathic differences in chemotherapeutic sensitization were observed in the caspase 3-reconstituted cells. To reflect *in situ* cell death in the original culture plates, the cells were treated at higher concentrations for a shorter period. When MCF-7/pv and MCF-7/c3 cells were treated with doxorubicin at concentrations of 0, 2.5, 5, and 10 μM or with etoposide at concentrations of 0, 100, 200, and 400 μM for 18 h, the differences between the two cell lines were evident at all doses. This effect was magnified at increased concentrations. Cellular alterations included shrinkage, rounding, detachment, membrane blebbing, and segregation of cellular structure. In the 10 μM doxorubicin (Fig. 2A)-treated group or 400 μM etoposide (Fig. 2B)-treated group, MCF-7/c3 cells displayed diffused apoptosis as compared with MCF-7/pv cells, which showed only sporadic islands of cell death.

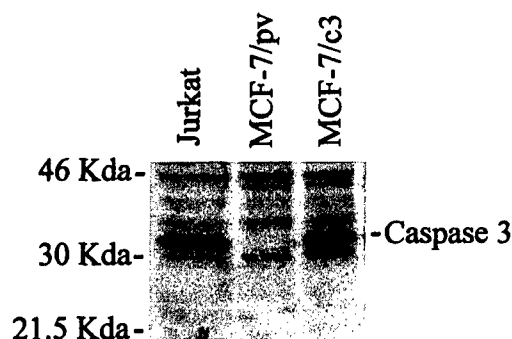


Fig. 1. Reconstitution of caspase 3 in MCF-7 cells. Protein levels of caspase 3 in Jurkat, MCF-7/pv, and MCF-7/c3 cells were detected using Western blot. MCF-7/pv and MCF-7/c3 cells were MCF-7 cells transfected with pBabe/puro vector and the vector encoding caspase 3 cDNA, respectively.

Table 1 Effects of doxorubicin and etoposide on survival fractions of MCF-7/pv and MCF-7/c3 cells

Treatment	MCF-7/pv		MCF-7/c3		P
	IC ₅₀ ^a	95% CI ^b	IC ₅₀	95% CI	
Doxorubicin (nM)	8.44	7.30–9.58	4.23	3.31–5.15	<0.01
Etoposide (μM)	0.36	0.19–0.53	0.09	0.08–0.10	<0.05

^a Mean of IC₅₀s. IC₅₀s were determined as described in "Materials and Methods." Five data sets were used for analysis.

^b CI, confidence interval.

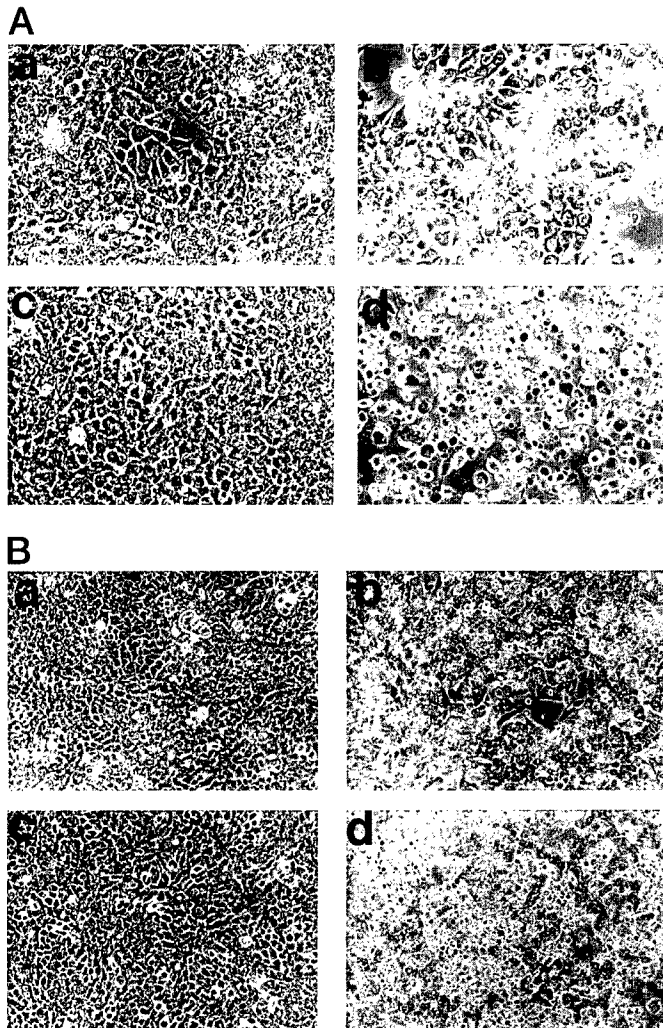


Fig. 2. Enhanced killing in MCF-7/c3 cells treated with doxorubicin (A) or etoposide (B). MCF-7/pv (A and B, a and b) and MCF-7/c3 (A and B, c and d) cells were treated with 10 μM doxorubicin (A, b and d) or 400 μM etoposide (B, b and d) for 18 h, as compared with untreated cells (A and B, a and c) cells. Photographs were taken under a phase-contrast microscope (10 × 20).

Activation of Effector Caspases in Caspase 3-reconstituted Cells. Activation of effector caspases is a biochemical hallmark of apoptosis. To verify that the above-described sensitization to the chemotherapeutic drugs occurred through caspase 3-mediated apoptosis, we analyzed the activation of effector caspases in MCF-7/c3 and MCF-7/pv cells. DEVD cleavage assay is a quantitative method that detects caspase 3-like activity (39). As shown in Fig. 3, DEVD cleavage activity in drug-treated MCF-7/pv cells was very limited, even in the cells treated with 5 μM doxorubicin or 200 μM etoposide. However, DEVD cleavage activity in MCF-7/c3 cells increased over 10–20-fold when the cells were treated with 200 μM etoposide or 2.5 μM doxorubicin. The strong caspase 3-like activity in drug-treated

MCF-7/c3 cells indicates that caspase 3 expressed in MCF-7/c3 cells was functional and that activation of reconstituted caspase 3 contributed to the sensitization.

To detect the activation of specific effector caspases, Western blots were performed. As indicated by decreased proform and subunit generation, activation of caspase 3 was detected in MCF-7/c3 cells treated with both drugs (Fig. 4, A and B). Although Western blotting was less sensitive than DEVD cleavage assay, the results obtained using either method were consistent with each other. Because caspases 6 and 7 are commonly activated in different apoptosis, we compared the extent and pattern of their activation between MCF-7/pv and MCF-7/c3 cells. In drug-treated MCF-7/pv cells, which were deficient of caspase 3, caspase 7 processing/activation was minimal (Fig. 4, A and B). In contrast, activation of caspase 7 in MCF-7/c3 cells was remarkably increased when the cells were treated with 10 and 50 μM doxorubicin or 200 and 400 μM etoposide, as indicated by the formation of p32 and p20 fragments. These results indicate that caspase 7 activation in doxorubicin- and etoposide-treated cells was primarily caspase 3 dependent. These observations were consistent with our reported finding of granzyme B-induced apoptosis (16).

Analysis of caspase 6 activation in the two cell lines revealed a more specific action of caspase 3. As shown in Fig. 4A, a p32 band product was identified in doxorubicin-treated MCF-7/pv cells but not in MCF-7/c3 cells, consistent with caspase 6 activation at low levels in the absence of caspase 3. Reconstitution of caspase 3, however, significantly enhanced caspase 6 activation at both 10 and 50 μM doxorubicin. Because the combined size of pLarge and pSmall subunits of caspase 6 is about M_r 32,000 (42), the appearance of a p32 band in MCF-7/pv cells suggests that caspase 6 was processed by a caspase other than caspase 3 between the propeptide and pLarge subunit. The disappearance of the p32 band and an increase in the pLarge subunit (p20) in treated MCF-7/c3 cells suggests that caspase 3 processes caspase 6 between the pLarge and the pSmall subunits. In etoposide-treated MCF-7/c3 cells, the extent of caspase 6 activation was not as great as that observed in doxorubicin-treated MCF-7/c3 cells (Fig. 4B). However, a cleavage product with a size around M_r 30,000 appeared specifically in etoposide-treated MCF-7/c3 cells. Disappearance of the p32 band in MCF-7/c3 cells treated with 800 μM etoposide also suggests the cleavage between pLarge and pSmall subunits. Taken together, these results support that activation of caspase 3, as well as the subsequent activation of caspases 6 and 7, contributed to the sensitization in MCF-7/c3 cells.

Cleavage of Cellular Death Substrates in MCF-7/c3 Cells. Because proteolytic cleavage of cellular death substrates by activated

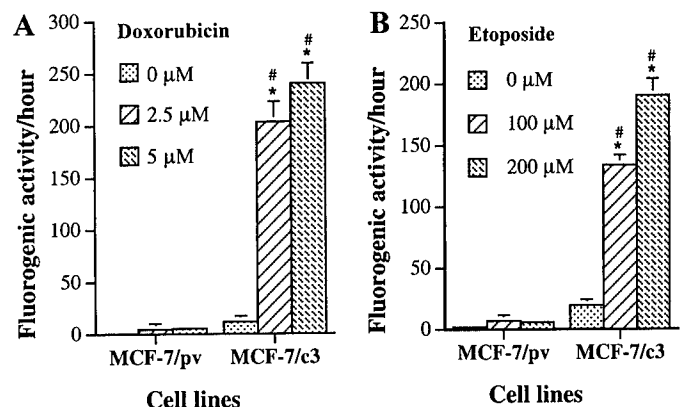


Fig. 3. DEVD cleavage activity in drug-treated MCF-7/pv and MCF-7/c3 cells treated with doxorubicin and etoposide. The cells were treated with doxorubicin (A) or etoposide (B) at the indicated concentrations for 18 h before the lysate was prepared for fluorogenic assay. *, $P < 0.005$ versus nontreated MCF-7/c3 cells; #, $P < 0.005$ versus the same treatment condition on MCF-7/pv cells.

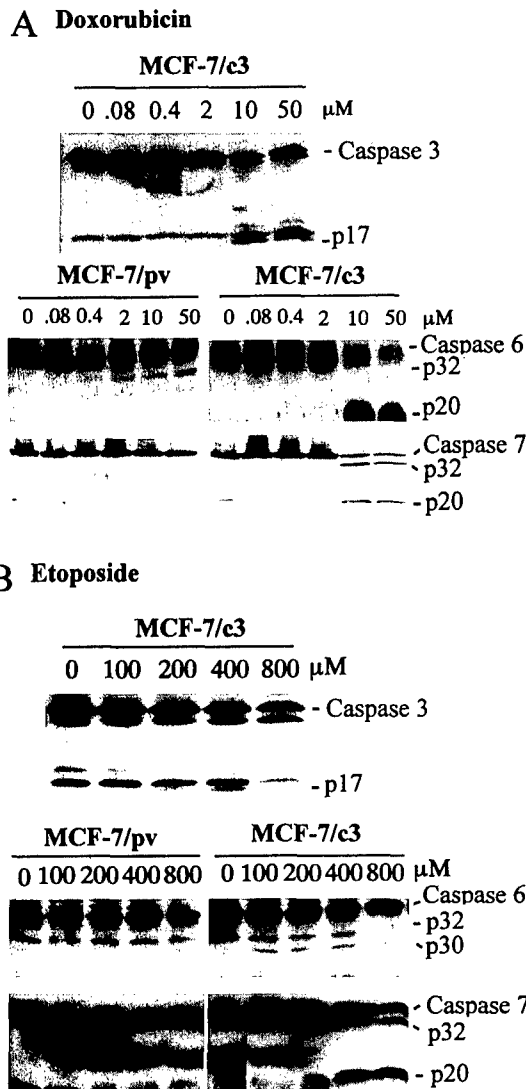


Fig. 4. Western blot showing the activation of caspases 3, 6, and 7 in doxorubicin- or etoposide-treated cells. The cells were treated with doxorubicin (A) and etoposide (B) at the indicated concentration for 18 h before the lysate was prepared for Western blot. Fifty μ g of lysate protein were separated with SDS-PAGE gel. The caspases were probed with specific antibodies against caspase 3, 6 and 7, respectively.

caspases is responsible for the cellular dysfunction and structural destruction of apoptosis (6), we studied the cleavage of PARP, lamin B, and DFF as representative substrates in the control and reconstituted cells. As shown in Fig. 5, there was only limited cleavage of all three substrates in MCF-7/pv cells treated with either drug (even when the doxorubicin concentration was as high as 50 μ M, and the etoposide concentration was as high as 400 μ M). In contrast, all three substrates were significantly or even completely cleaved in the drug-treated MCF-7/c3 cells. These results are further evidence supporting a pivotal role for caspase 3 in chemotherapy-induced apoptosis.

Caspase 3 Was Required for Doxorubicin- and Etoposide-induced DNA and Nuclear Fragmentation. DNA and nuclear fragmentation is a key feature associated with apoptosis (6). Caspase 3 has been reported to be required for DNA fragmentation in tumor necrosis factor α -induced apoptosis (35). To examine the effect of caspase 3 reconstitution on DNA fragmentation and nuclear morphology in doxorubicin- and etoposide-induced apoptosis, we analyzed these changes in drug-treated MCF-7/c3 cells and control MCF-7/pv cells. Flow cytometry analysis detected significant DNA fragmentation (the hypodiploid peak) only in drug-treated (doxorubicin or etoposide)

MCF-7/c3 cells (Fig. 6, A and B). Nuclear morphology corresponding to DNA fragmentation was verified using DAPI staining of the treated cells. In contrast to drug-treated MCF-7/pv cells, which only displayed nuclear condensation in apoptotic cells, apoptotic MCF-7/c3 cells showed typical nuclear fragmentation (Fig. 6C). These results suggest that caspase 3 was also required for DNA and nuclear fragmentation in chemotherapy-induced apoptosis.

DISCUSSION

In this report, we describe the establishment of a stable MCF-7 cell line reconstituted with caspase 3. This line was useful for studying the specific role of caspase 3 and caspase 3-dependent signaling in response to doxorubicin and etoposide. As demonstrated by IC_{50} determination and morphological data, caspase 3 reconstitution sensitized MCF-7 cells to doxorubicin- and etoposide-induced apoptosis. Increased DEVD cleavage and amplified activation of caspases 6 and 7 were also observed after treatment in caspase 3-reconstituted cells. Significant increases in the proteolysis of cell death substrates and DNA fragmentation further verified a caspase 3-mediated sensitization in doxorubicin- and etoposide-induced apoptosis.

Doxorubicin and etoposide are active chemotherapeutic agents used in clinical oncology. Doxorubicin is a key adjuvant drug for breast cancer treatment. It triggers apoptosis through several mechanisms. As with many chemotherapeutic agents, it induces DNA damage by interacting with topoisomerase II, leading to DNA breakage (43). It can also induce membrane alterations and the generation of ceramide at higher concentrations (44). Recently, it has been reported that up-regulation of the Fas/Fas ligand system may also be involved in doxorubicin-mediated killing (45). For etoposide-induced apoptosis, DNA damage secondary to topoisomerase II inhibition appears to be

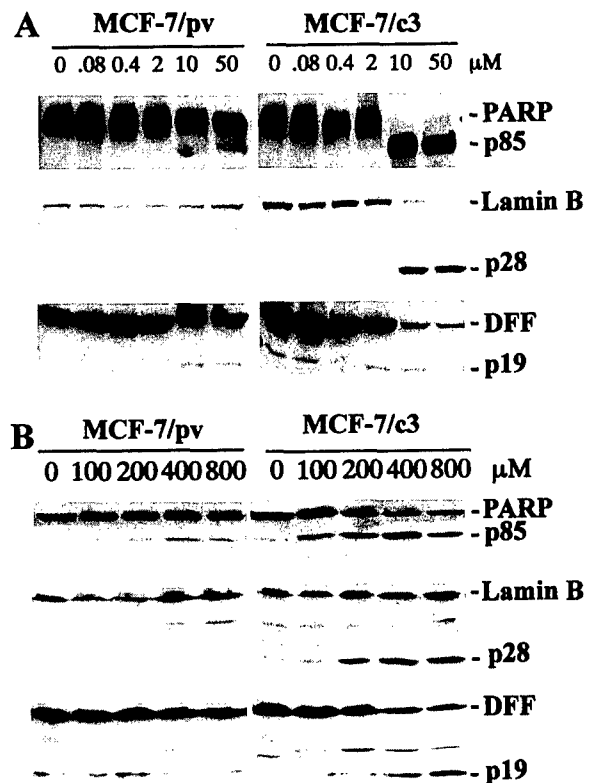


Fig. 5. Proteolytic cleavage of PARP, lamin B, and DFF in doxorubicin (A)- and etoposide (B)-treated cells. The conditions for sample preparation and Western blot were the same as those described in the Fig. 4 legend. Cleavage of PARP, lamin B, and DFF was detected with the corresponding specific antibody, respectively.

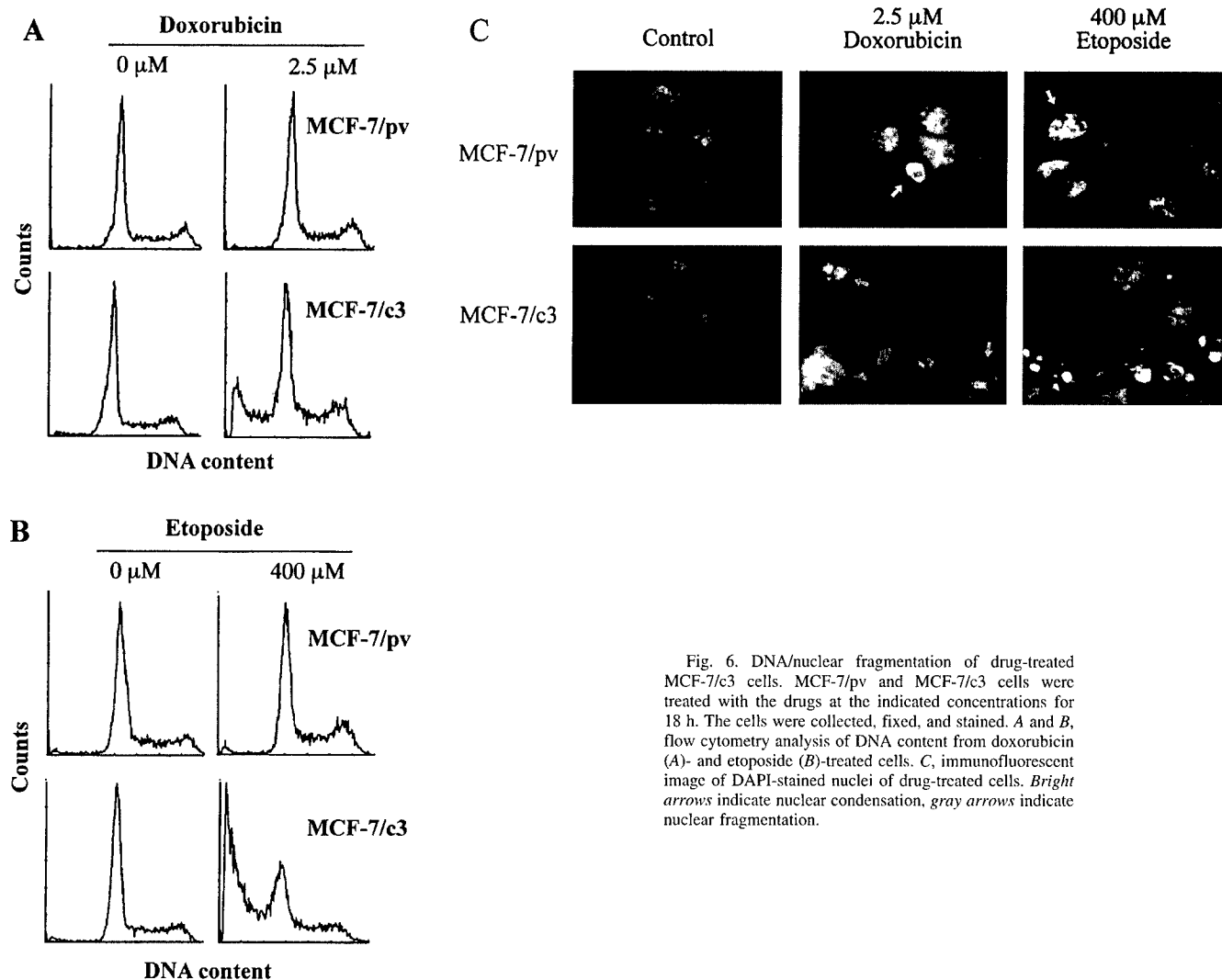


Fig. 6. DNA/nuclear fragmentation of drug-treated MCF-7/c3 cells. MCF-7/pv and MCF-7/c3 cells were treated with the drugs at the indicated concentrations for 18 h. The cells were collected, fixed, and stained. A and B, flow cytometry analysis of DNA content from doxorubicin (A)- and etoposide (B)-treated cells. C, immunofluorescent image of DAPI-stained nuclei of drug-treated cells. *Bright arrows* indicate nuclear condensation, *gray arrows* indicate nuclear fragmentation.

a major mechanism (46). Despite the variance in the chemotherapeutic initiation process, the resulting release of cytochrome *c* from mitochondria followed by activation of caspase 9 and the effector caspases is believed to be the final common pathway in chemotherapy-induced cell death (38, 47, 48). Microinjection of cytochrome *c* induced apoptosis in 293 cells with functional caspase 3 or caspase 3-transfected MCF-7 cells but not in caspase 3-deficient MCF-7 cells (36), indicating that caspase 3 was required for cytochrome *c*-mediated apoptosis. Abrogation of mitochondrial cytochrome *c* release and caspase 3 activation have been associated with acquired multidrug resistance (49). As shown in this presentation, caspase 3 reconstitution restored the integrity of the doxorubicin- and etoposide-induced killing mechanism. This direct evidence links caspase 3 deficiency and chemotherapeutic efficacy, suggesting caspase 3 defects as one mechanism for chemoresistance.

Activation of caspase 3 in chemotherapy-induced apoptosis has been reported by many groups (17, 38, 47, 50, 51). Involvement of caspase 3 in this process was shown either by detecting its activation as a representative of effector caspases (38, 50, 52) or by using synthetic inhibitors, such as DEVD-CHO (51, 53), to block caspase 3-like activities. Nevertheless, little work has been done to differentiate the role of caspase 3 from that of other effector caspases in this process. In our experiments, comparison between caspase 3-deficient and -reconstituted cell lines more specifically defined the specific role of caspase 3 in doxorubicin- or etoposide-induced apoptosis and in the

activation of other effector caspases. Although caspases 3, 6, and 7 are all categorized as effector caspases (4, 5), our results demonstrated an additional apical-like nature of caspase 3. These data, derived from a whole cell system (in contrast to a cell-free system), show that activation of caspase 6 and especially caspase 7 was largely dependent on caspase 3 activation (Fig. 4). Although caspase 6 activation was detected in caspase 3-deficient cells, efficient activation of caspase 6 required caspase 3 activity (Fig. 4). As a result, by direct cleavage and amplification through the activation of other effector caspases, caspase 3 reconstitution led to a striking increase in death substrate cleavage and DNA fragmentation (Figs. 5 and 6). Our preliminary results showed that reconstituted caspase 3 also had feedback effects on its upstream factors.⁴

Although our experiments were based on an *in vitro* cell line model, our data are consistent with a recent report that was based on an *in vivo* model. Using a rat AH130 liver tumor model, Yamabe *et al.* (54) found that transduction of human caspase 3 in combination with etoposide administration induced extensive apoptosis and significantly reduced tumor volume, as compared with the group with caspase 3 transduction or etoposide treatment alone. Although our focus was on reconstitution of caspase 3, and theirs was on caspase 3 overexpression-mediated therapy, both reports demonstrate that

⁴ Unpublished observations.

caspase 3 is critical in chemotherapy-induced apoptosis and that caspase 3 reconstitution/overexpression and chemotherapy have synergistic effects.

In our experiments, two drug treatment conditions were used. For IC₅₀ determination using MTT assays, the cells were exposed to the drugs for 6 days. Significant sensitization was detected when drug concentrations were between 0.5 and 37 nM for doxorubicin and 0.05 and 1 μ M for etoposide, respectively. This compares favorably with the plasma concentrations of the two drugs in clinical application, which could reach up to 2 μ M for doxorubicin (55) and 170 μ M (100 μ g/ml) for etoposide (56), respectively. To evaluate early biochemical changes, the cells were also treated for shorter periods (18 h) at much higher concentrations, although this very high dose and short duration approach is not currently clinically feasible.

One noteworthy finding was that when MCF-7/c3 cells were treated with doxorubicin for 18 h, caspase activation and death substrate cleavage displayed a sharp increase when the drug concentration was increased from 2 to 10 μ M (Figs. 4 and 5). This suggests that there may be a concentration threshold for doxorubicin to induce maximal caspase 3-mediated apoptosis under a given treatment condition. This is consistent with the clinical benefit observed with dose intensification of doxorubicin, as shown for node-positive breast cancer patients (57) and topical administration of doxorubicin for ovarian cancer (58). Because significant sensitization to doxorubicin was also detected in MTT assays, we agree with Han *et al.* (59) on the action model of doxorubicin. Doxorubicin appears to induce two types of cellular response, *i.e.*, slow cell death at low concentrations and rapid cell death at high concentrations. This may be due to an increased number of activated mechanisms at higher drug concentrations.

Distinct differences in apoptotic activities between MCF-7/pv and MCF-7/c3 cell lines in response to doxorubicin or etoposide treatment underscore the possible significance of caspase 3 deficiency in cancer resistance. Caspase 3 reconstitution sensitized MCF-7 breast cancer cells to commonly applied chemotherapeutic agents, suggesting that caspase 3 deficiency may contribute to chemotherapeutic resistance. Caspase 3 reconstitution also sensitized MCF-7 cells to radiotherapy and granzyme B (16).⁴ Reconstitution of caspase 3 in MCF-7 cells may also enhance apoptosis in response to Fas ligand and tumor necrosis factor α treatment, as shown by others (35, 37). Therefore, it appears that caspase 3 deficiency may have a broad clinical relevance, including both chemo- and radiotherapeutic resistance and immune-associated antitumor mechanisms. Our preliminary results show down-regulation or deficiency of caspase 3 in many breast cancer specimens,⁴ which supports the non-breast cancer findings of others (60). We therefore speculate that caspase alterations may be linked to poorer prognosis and therapeutic resistance in human breast cancer.

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